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THE UNIVERSITY OF ALBERTA

MOLECULAR AND FUNCTIONAL ANALYSIS OF CD45
IN T CELL DEVELOPMENT

by

Julie P. Deans

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
Doctor of Philosophy

MEDICAL SCIENCES (IMMUNOLOGY)

EDMONTON, ALBERTA
SPRING 1990

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J Deans

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Date: 21 December, 1989

Then I heard the now-familiar honking of the geese flying over the summit ridge at more than 25,000 feet........................

..........In a sense we were like them. In this high, confined world, with no sound but the wind and our own voices; with no sights but the blue sky, white peaks, and bright colors of our tents and parkas; with no sensations but the cold of the ice and snow and the warmth of the sun, our sleeping bags, and stoves; we had a chance to slow our world down, to examine our world and our purpose. An important question, as always, was why were we here? Answers came readily: to visit Asia, climb a mountain, test our limits, know ourselves. All these were true, yet they were not enough. Why would any woman risk her life to stand on the top of a mountain? The geese circled the summit once before resuming their flight south. Were they wheeling among the high peaks for the view? For the glory? I smiled and thought, "I bet they're doing it for the fun of it."

from "*Annapurna: A Woman'sPlace*"
by Arlene Blum.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

MOLECULAR AND FUNCTIONAL ANALYSIS OF CD45
IN T CELL DEVELOPMENT

submitted by JULIE P. DEANS

in partial fulfilment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

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ABSTRACT

CD45 is a family of leukocyte transmembrane glycoproteins with tyrosine phosphatase activity in the cytoplasmic domains, and extracytoplasmic variations created by alternate splicing of three exons. Terminal differentiation of T cells, from antigen-inexperienced to memory T cells, is associated with a shift in expression of CD45 from high (CD45RA, p220) to low (CD45R0, p180) m.w. isoforms. In this thesis the kinetics of the cell-surface transition has been analyzed and correlated with mRNA synthesis. We found that after 4 h of mitogen-stimulated activation high m.w. CD45 mRNA increases significantly above resting-state levels, but then declines within 24 h. CD45R0 mRNA is detectable by 24 h post-stimulation, indicating a rapid switch in CD45 mRNA splicing patterns. The $t_{1/2}$ of mRNA encoding high and low m.w. isoforms was estimated at 2.25 h and 3.5 h, respectively.

We have proposed an essential role for CD45RA in intrathymic development, postulating that expression of CD45RA defines the intrathymic generative lineage. In support of this hypothesis we have shown that the majority of $CD3^-4^-8^-$ thymocytes are $CD45RA^+$, and that antibodies to CD45RA stimulate IL-2 and IL-2R mRNA synthesis by $CD3^-4^-8^-$ thymocytes, in the presence of suboptimal doses of PHA and PMA. Maximal proliferation of these cells was achieved by induction through CD2 and CD28, as well as CD45RA. In immature thymocytes activated with CD2 and CD28 antibodies, the level of CD45 mRNA remained high even at 48 h, in contrast to mitogen-activated mature peripheral T cells. The results demonstrate that CD45RA transmits activation signals to immature thymocytes, and support the hypothesis that CD45RA expression is essential for cell survival in the thymus. We further propose that continuous expression of CD45RA on thymocytes depends on activation via CD28.

PREFACE

In the years since they were first described, the CD45 family of cell surface molecules has been referred to by a variety of names. The nomenclature is presently undergoing a rapid phase of evolution, as recent developments have clarified the nature of the polypeptide differences between the family members, and the location of the epitopes recognized by certain of the antibodies has been determined. At the 4th International Workshop on Human Leucocyte Differentiation Antigens, held in Vienna in February 1989, the nomenclature was adjusted to facilitate description of the molecules as we now know them and to provide flexibility for future naming of family members as antibodies become available to distinguish between them. The term "CD45", unembellished, refers to the whole family of molecules, and "antibodies against CD45" can be assumed to recognize determinants which are common to all of them. When "CD45" is followed by the letter "R" a reference is being made to a phenotypically restricted form of CD45, as defined by reactivity with antibodies that have restricted specificity within the CD45 family. The precise restricted form is indicated by the letter following the "R", for example "CD45RA".

There are potentially eight members of the CD45 family of molecules, produced by alternate splicing of three exons near the 5' end of the primary RNA transcript. When none of these exons is included in the final mRNA product, a CD45 molecule is produced which is now referred to as CD45R0. Previously, CD45R0 was called CD45 p180. When the most 5' of the three variable exons, originally named exon A, is included, the protein product is recognized by antibodies such as 2H4 and 3AC5, and referred to as CD45RA. CD45RA was previously known simply as CD45R, or as CD45 p220/205. CD45RA and CD45R0 are the isoforms of CD45 which are best characterized at the present time, due to the availability of identifying antibodies. Wherever possible, the new nomenclature will be adhered to in the following pages.

The work described herein was done exclusively on human tissue. Although CD45 has been studied extensively in the rat and mouse, in some respects the characterization of CD45 in the human has been more revealing, due to the fortuitous development of antibodies which distinguish some of the major isoforms. For this reason, the following literature review emphasizes work done using human cells, although not to the exclusion of the large body of data from rat and murine studies. For example, ultrastructural studies of CD45 have been done only in the rat and due reference is made to that work, and to other studies using non-human tissue, whenever it is additive and clarifying.

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There are many people who have contributed, in one way or another, to this achievement. Above all, I formally thank my parents, Myra and Robert Deans, for providing me with an excellent early education, and for their moral and financial support through my undergraduate years.

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I am most indebted to Dr. Vern Paetkau for allowing me to work in his lab for many months, and to Dr. Jennifer Shaw who patiently taught me the fundamentals of molecular biology, and guided much of my work in this area. Thanks also to Rosemary Garner who coped cheerfully with the intrusion and my many pesky questions.

The time that I spent at the Walter and Eliza Hall Institute for Medical Research, Melbourne, was productive and exciting, and also the most enjoyable phase of my graduate work. For that experience I am indebted to Dr. Ken Shortman, Dr. Martin Pearse, Wu Li, Nella Fisicaro, Anne Wilson, and to my good friends Lesley Rogers, James, Jean-Luc and Uli.

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My decision to undertake graduate training in Immunology was one that evolved over some time. I was positively influenced by Dr. Robert Lannigan, who first introduced me to Immunology research, and by Bobbie Liles; both of whom, for many years, had more faith in my abilities than I did myself. Their encouragement and support, as well as that of Dr. Betsy Gard, were crucial to my decision. I also warmly thank Sig Olson, and my colleagues at the American Red Cross in Atlanta for their friendship and support.

During the period of my Ph.D. candidacy, I have been buoyed by the love and support of my sister, Ann, my brother, Alan, and our parents. I am also endlessly appreciative of Jennifer, for her patience, perspective, good humor, encouragement and understanding, particularly during the writing of this thesis, and for sharing all the joys and trials.

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Finally, I am pleased to acknowledge my receipt of Studentship Awards from the Alberta Heritage Foundation for Medical Research.

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LIST OF ABBREVIATIONS

Ag	Antigen
CsA	Cyclosporin
Cx	Cycloheximide
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
IF	Immunofluorescence
Ig	Immunoglobulin
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
IL-7	Interleukin 7
mAb	Monoclonal antibody
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
PTPase	Protein tyrosine phosphatase
TCR	T cell receptor for antigen

I. HYPOTHESIS AND REVIEW OF THE LITERATURE RELATING TO CD45

Intrathymic events governing the proliferation, differentiation and fate of developing thymocytes are poorly understood. It is known that a small number of bone marrow-derived stem cells seed to the thymus (1,2) and that their earliest identifiable pre-T cell progeny, CD2$^+$ CD3$^-$ CD4$^-$ CD8$^-$ cells, proliferate rapidly and give rise to all other thymocyte subsets (2-5). The vast majority of thymocytes die intrathymically (6-8), presumably as a result of either selection or the occurrence of non-functional T cell antigen receptor (TCR) rearrangements on both chromosomes. Those cells which survive and emerge from the thymus to populate the peripheral T lymphocyte pool represent about 1% of all thymocytes (7).

There is at present no phenotypic criterion that allows the identification of cells within the generative lineage, distinguishing them from cells which are destined for intrathymic death. We have proposed (9,10) that CD45RA molecules provide such a marker, based on the following observations: 1) 99% of T cells isolated from 17-24 week human fetuses are CD45RA$^+$ (11); 2) 95% of T cells in neonatal cord blood are CD45RA$^+$ (12,13); 3) The proportion of CD45R0$^+$ cells increases with age (14); 4) The 60% of adult peripheral blood T cells which are CD45RA$^+$ appear to be the most immature, antigen-inexperienced set, giving rise to the reciprocal CD45R0$^+$ memory T cells (14,15); 5) Although the majority of thymocytes are CD45R0$^+$ (16,17), CD45RA$^+$ cells are represented in all thymic subsets defined by CD3, CD4 and CD8 (9,10), and are particularly abundant among cells of the least mature (CD3$^-$4$^-$8$^-$) and most mature (CD3$^+$4$^+$8$^-$ and CD3$^+$4$^-$8$^+$) subsets. Taken together, these observations imply that either CD45RA is acquired as a post-thymic differentiative step on those thymic emigrants which are CD45RA$^-$, or only those thymocytes which are CD45RA$^+$ ever emerge from the thymus.

In the periphery the CD45RA$^+$ and CD45R0$^+$ phenotypes are largely non-overlapping subsets (13,14,18-20), initially thought to belong to separate lineages defining suppressor-inducer T cells and helper T cells respectively (21,22). It now appears, however, that they are linearly related in a uni-directional differentiative process, since CD45RA$^+$ cells become irreversibly CD45R0$^+$ following *in vitro* activation (10,14,20,23-27). There is at present no evidence to suggest that CD45R0$^+$ cells can acquire CD45RA (20,24,28). It therefore seems more likely that thymic emigrants are CD45RA$^+$ than that they should acquire CD45RA post-thymically. Similarly, to the extent that it is possible to extrapolate this reasoning to cells differentiating within the thymus, it seems more likely that thymocytes within the generative lineage are CD45RA$^+$ from the earliest precursor stage, and remain so until they exit from the thymus, than that they should either lose and later reacquire its expression, or start out as CD45RA$^-$ and acquire CD45RA at a later stage.

Support for the proposal that thymic migrants are CD45RA$^+$ comes from clonal analysis at limiting dilution of thymic subsets depleted either of CD45RA$^+$ cells or CD45R0$^+$ cells (9). It was demonstrated that all of the cells in the thymus with clonogenic potential could be accounted for in the CD45R0-depleted (CD45RA-enriched) population (9). Work described herein provides additional supportive evidence for the hypothesis that cells within the generative lineage of thymocytes are exclusively CD45RA$^+$, and that acquisition of CD45R0 is part of the mechanism of intrathymic cell death.

CD45

CD45, also known as T200, L-CA (leukocyte common antigen) and Ly-5, is a series of transmembrane glycoproteins uniquely expressed in some form on all cells of both myeloid and lymphoid lineages (29-37). CD45 represents a significant proportion, around 10%, of all lymphocyte surface molecules, and is heavily glycosylated, consisting of about 25% carbohydrate by weight (29,38). CD45, in fact, carries most of the membrane-associated carbohydrate (29). Immunoprecipitation of CD45 from polyclonal populations, using antibodies which recognize common determinants on CD45, reveal four predominant isoforms, varying in molecular weight between 180 kD and 220 kD (39). Variations in size are due primarily to differences in the length of the polypeptide backbone (40), and secondarily to differential glycosyations which may be cell type-specific (41).

Genomic Organisation: CD45 isoforms are encoded by a single gene of 33 exons spanning over 130 kb (29,42,43) on chromosome 1 (44). Exon 1 codes for the 5' untranslated region of the mRNA, exon 2 encodes the signal peptide, exons 3-15 encode the region of the molecule which is external to the cell, exon 16 encodes the transmembrane domain, and exons 17-32 encode the cytoplasmic region (45). Exon 33 codes for the 3' end of the cytoplasmic domain, as well as the 3' untranslated region of the mRNA (45).

All isoforms of CD45 are translated from mRNA's which have in common exons 3, and exons 7-33 (45). Exons 4, 5 and 6 are alternatively spliced from the primary transcript to create potentially eight different mRNAs (45-47). At least 5 of these are known to exist in humans (45) and a sixth has been found in mice and rats (46,47) (Fig. 1.1).

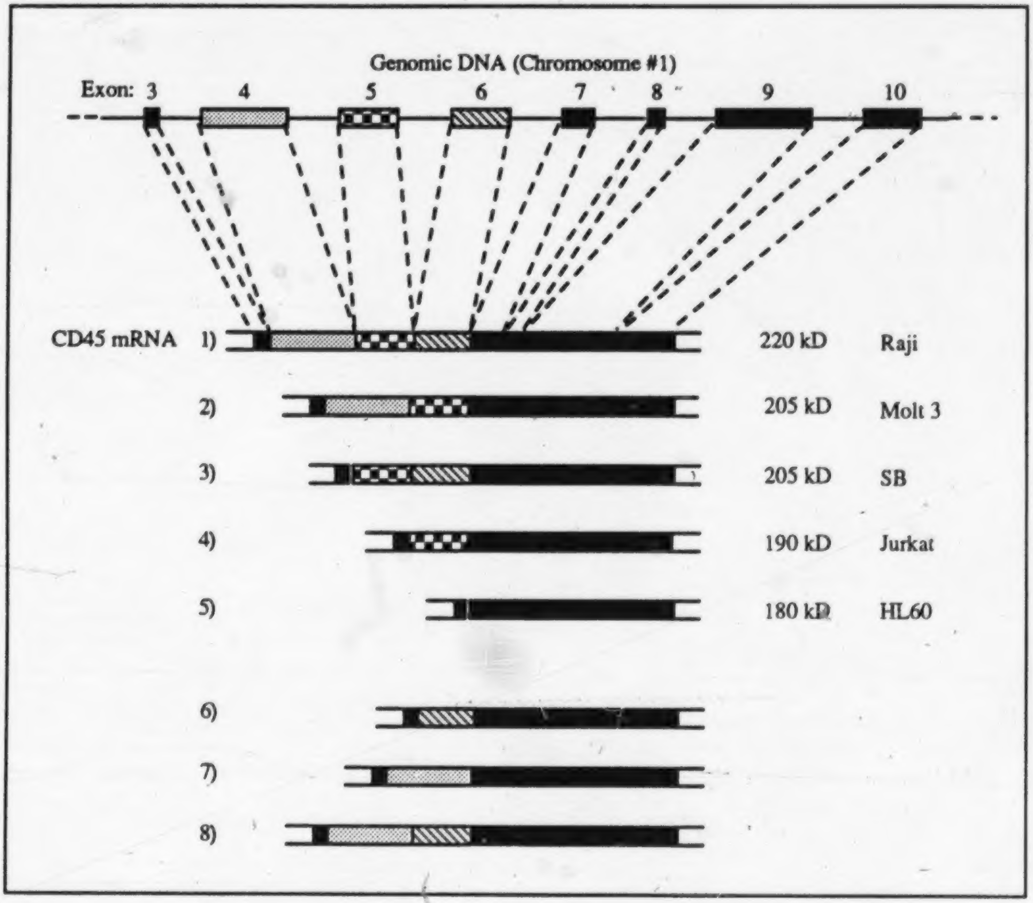

Genomic DNA (Chromosome #1)
Exon: 3
4
5
6
7
8
9
10
CD45 mRNA
1)
2)
3)
4)
5)
6)
7)
8)
220 kD
205 kD
205 kD
190 kD
180 kD
Raji
Molt 3
SB
Jurkat
HL60

Figure 1.1. Schematic diagram of the generation of alternate isoforms of CD45 by differential usage of exons 4, 5 and 6. Boxes represent exons, drawn approximately to scale. Lines indicating introns in the genomic map are not drawn to scale. Solid boxes are invariant. CD45 mRNAs 1-5 have been identified in humans; a sixth, including exon 6 but lacking exons 4 and 5, has been observed only in mice and rats. Combinations depicted in 7 and 8 have not yet been reported. Listed on the right are the sizes of the respective glycoproteins and examples of cell lines that express predominantly the CD45 mRNA indicated.

Regulatory mechanisms controlling alternative splicing of the CD45 primary transcript are not known in detail, however it has been reported that regions within exons 4, 5 and 6, and in their immediate flanking introns, are essential, whereas regions in adjoining exons 3 and 7 are not required (48). While alternative splicing is a common method of producing variations of the same protein (49), the splicing pattern for CD45 is unusual in three respects (45). First, the potential for 8 different forms is a relatively high degree of diversity; it is more common for alternative splicing to produce 2 or 3 different forms. Second, the diversity involves the N-terminus, whereas many alternatively spliced mRNAs produce proteins with differences at the C-terminus, for example, secreted versus membrane-bound forms of the same molecule. Third, the splicing patterns are developmentally regulated rather than random (45).

Cytoplasmic Region: Structure and Function: The cytoplasmic region of the CD45 glycoprotein is 707 amino acids in length (44), the largest of any transmembrane molecule characterized to date (Table 1.1). The entire length of the cytoplasmic domain is highly conserved between species, with 85% homology of amino acids between mice and humans, rising to 95% when conservative substitutions are taken into account (29). Exons encoding the cytoplasmic region fall into 2 groups, 17-24 and 25-33 (43), with approximately 33% homology to one another (42). Until recently these domains showed no homology to any known protein, including other transmembrane proteins with which CD45 shares the property of having very large domains on both sides of the plasma membrane (Table 1.2). Some of these molecules are known to be receptors with kinase activity in their cytoplasmic domains (50). Recently, a protein tyrosine phosphatase (PTPase) was purified to homogeneity from human placenta (51,52). A search of the protein sequence data base using the amino acid sequence obtained, revealed significant homology to the cytoplasmic domains of CD45 (53). Partial amino

TABLE 1.1

Comparison of the lengths of cytoplasmic domains of several transmembrane molecules

Transmembrane molecule	**Cytoplasmic region**
IgM and IgD heavy chains	3*
TCR-α and TCR-β	5
Class I heavy chain	31
Class II α and β chains	15, 16
CD3	44
IL-2 receptor	13
Transferrin receptor	62
EGF receptor	542
Insulin receptor	402
N-CAM	362
CD45	707

* Number of amino acids

TABLE 1.2

CD45 shares overall structural similarity with several other transmembrane proteins

	Domain		
Protein	**External**	**Transmembrane**	**Cytoplasmic**
EGF-R	621*	20	542
PDGF-R	500	25	542
Insulin R	930	22	403
neu	640	19	580
c-fms	512	23	337
N-CAM	584	18	362
CD45	391-552	22	707

* Number of amino acids

acid sequence of CD45 had been obtained directly (54), confirmed and extended by deduction from cDNA sequencing (45). Subsequently, PTPase activity has been assigned directly to purified CD45 (55-57).

Tyrosine phosphorylation is thought to be important in the control of cell growth and differentiation, since several growth factor receptors are tyrosine kinases, and a number of transforming retroviruses carry homologues of cellular genes with tyrosine kinase activity (57). Thus, the identification of CD45 as a tyrosine phosphatase implicates it in the regulation of cell growth and differentiation. The T cell specific tyrosine kinase $pp56^{lck}$ has been the subject of much interest recently, since it was shown to be physically associated with cytoplasmic domains of CD4 and CD8 (57-61), suggesting a role in antigen-specific T cell responses. Activation of $pp56^{lck}$ is associated with the dephosphorylation of a tyrosine residue near the N-terminus. Recently, it has been reported that $pp56^{lck}$ cannot be activated in a mutant BW5147 T lymphoma line lacking CD45 (62), and furthermore, isolated CD45 directly activated $pp56^{lck}$ in immunoprecipitates of the two proteins (62). The identification of $pp56^{lck}$ as a substrate for the enzymatic activity of CD45 suggests that CD45 has an important role in T cell activation.

The cytoplasmic region of CD45 is itself a substrate for protein kinase C (63) and is rapidly phosphorylated following activation of T cells with phorbol esters (64). There are multiple potential phosphorylation sites, primarily on serine residues, and the CD45 molecules of the murine thymoma cell line BW5147 have been shown to be constitutively phosphorylated on serine (65). CD45 molecules appear to be linked to the cytoskeleton in a 1:1 molar complex with fodrin (66), which is associated with the actin/myosin component of the cytoskeleton. CD45 molecules may therefore have a role in the mobilization of other cell surface molecules with which it is associated. How this function relates to the phosphatase activity is presently unknown. Recently, Marano et al. (67) reported that the TCR complex associates with the cytoskeleton after TCR cross-linking by anti-CD3: it is tempting to speculate that the association of TCR with the cytoskeleton may be mediated by CD45.

Extracytoplasmic region: Antigenic variations and size differences among the CD45 isoforms are created by the pattern of expression of the three spliceable exons (44) (Fig.1.1). Exon 4 encodes 66 amino acids, exon 5 encodes 47, and exon 6 encodes 48 amino acids (44,45). When all 3 exons are included in the mature mRNA, the full-length glycoprotein of 220 kD is produced (44). The absence of all 3 results in the 180 kD isoform. The presence of 1 or 2 exons yields the intermediate isoforms of 190 kD and 205 kD, respectively (Fig.1.1).

Electron microscopy studies of affinity purified CD45 molecules, visualized by low angle shadowing, has revealed that while the cytoplasmic domains assume a globular configuration, the external region has a rod-like shape, estimated at 18 nm in length, with its long axis lying parallel to the plasma membrane (68) (Fig. 1.2). The amino acid sequence of the external region encoded by exons 7-15, includes 16 cysteine residues (45,54), providing potential for up to eight stabilizing disulphide bonds in the rod-shaped portion of the molecule. The variable part of the molecule, which lies 8 amino acids short of the N-terminal, contains no Cys residues, remarkably few hydrophobic residues, and is rich in Ser, Thr and Pro (44) (Table 1.3), suggesting an extended configuration with little secondary structure. These properties are also characteristic of regions containing O-linked glycosylation sites (29,69). The most extensive characterization of the glycoprotein has been done on the rat CD45R0 molecule, where

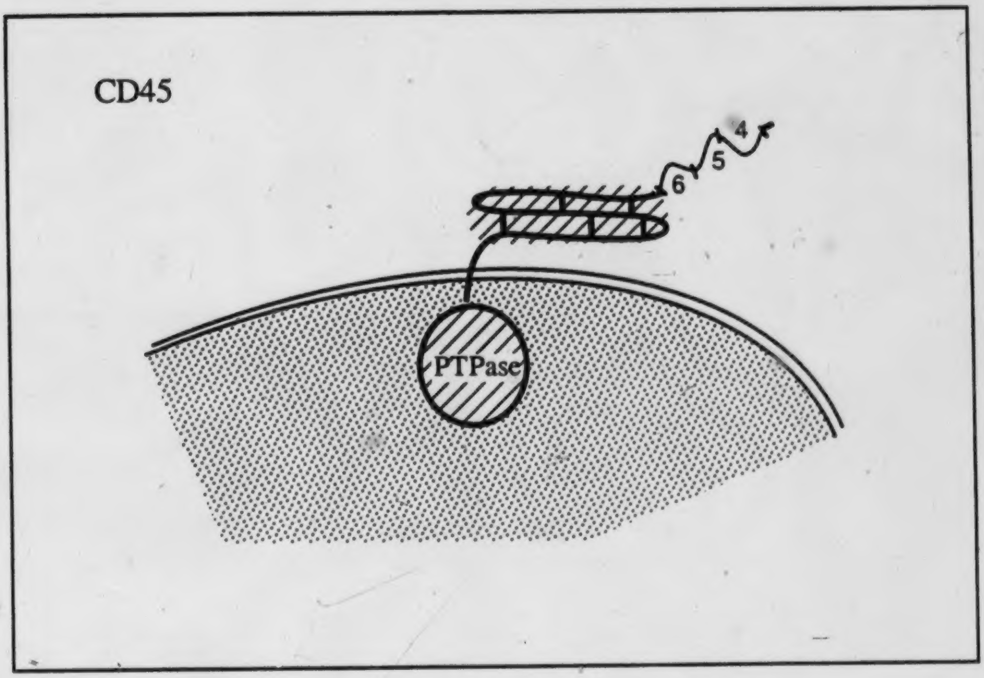


Figure 1.2. Schematic diagram of CD45 transmembrane glycoprotein. The cytoplasmic domain with PTPase activity is connected by a hydrophobic transmembrane stretch to the extracytoplasmic region. Amino acid sequences shared by all CD45 isoforms are indicated with a bold line. Vertical lines connecting the loops illustrate disulphide bonds. Variable sequences encoded by exons 4-6 contain no Cys residues, and is rich in Ser, Thr and Pro, suggesting extended configuration with little secondary structure.

TABLE 1.3

Comparison of the potential carbohydrate attachment sites and disulphide-bonds in the N-terminal (exons 3-8) and membrane-proximal (exons 9-15) regions of CD45

Exon(s)	Number of amino acids	Cys	Ser[a]	Thr[a]	Pro[a]	Potential O-linked sites	Potential N-linked sites
3	8	0	2	2	3	4	0
4	66	0	12	13	7	25	3
5	47	0	8	7	5	15	0
6	48	0	8	11	5	19	2
7	25	0	5	6	1	11	1
8	9	1[b]	1	3	2	4	0
3-8	203	1	36	42	23	78 (38%)	6
9-15	349	15	21	29	16	50 (11%)	10

[a] Amino acid sequences that are rich in serine, threonine and proline residues are characteristic of O-linked glycosylation sites.

[b] The cysteine residue coded for in exon 8 is the most C-terminal residue of the O-linked domain (exons 3-8), representing the beginning of the cysteine-rich region.

it has been demonstrated that all of the O-linked carbohydrate is located in the first 32 amino acids (70), corresponding to exons 3 and 7 (Table 1.3). Higher molecular weight forms would be expected to carry additional O-linked carbohydrate. Although the significance of the glycosylations on CD45 are presently unknown, it seems likely that O-linked carbohydrate moieties are involved in ligand binding. CD45, for reasons to be discussed below, is thought to be a receptor interacting with other cell surface molecules on the same and/or different cells (29). If this is correct there are probably several ligands for CD45, accounting for the variability in protein sequence and accompanying carbohydrate groups at the N-terminal.

Antibodies to CD45 fall into two categories: those which recognize common determinants on all CD45 molecules, and those with specificities restricted to particular isoforms of CD45. Of the latter group, well-characterized antibodies are available which define 3 of the 6 reported isoforms. The antibody UCHL1 defines the p180 isoform (18,19), now known as CD45RO. At present, UCHL1 is the only antibody of its kind, presumably recognizing an epitope formed by the joining of sequences encoded by exons 3 and 7. There are now several examples of antibodies which recognize epitopes on the sequence encoded by exon 4 (71,72). Exon 4 has been found in combination with exon 5, creating one of the p205 isoforms, and with both exons 5 and 6, creating the largest of the CD45 isoforms, p220 (Fig. 1.1). These antibodies, therefore, detect p220 and one of the possible p205 molecules, and together these isoforms constitute CD45RA. Consistent with this is the finding that anti-CD45RA antibodies precipitate molecules of these sizes from the surfaces of radiolabelled lymphocytes (16,21,26). The two potential species of CD45 mRNA's which have not been found are those in which exon 4 would be included alone or with exon 6.

There are recent reports of the production of antibodies with additional restricted specificities (16,73), but these are still in the process of characterization (72,74). In addition, three antibodies which detect carbohydrate epitopes on CD45 molecules have been described (75,76): CT1 and CT2 antibodies recognize an O-linked carbohydrate structure expressed on murine fetal thymocytes (41) and CTL's (76); the NK-9 antigen is an O-linked carbohydrate epitope expressed on 85-90% of all human T cells and NK cells (75).

Differential expression of CD45 isoforms: The expression of CD45 is limited to cells of the lymphoid and myeloid lineages. Among the different cell types which do express CD45 there is considerable variability in isoforms expressed and in the cell-surface density of the antigens. Granulocytes, neutrophils and macrophages are reported to be predominantly CD45R0$^+$ (16,20,22,26,39,77), and express the antigen at a significantly lower density than do lymphocytes (78). NK cells express higher molecular weight forms of CD45 (29,75). The majority of B cells bear CD45RA, with different glycosylations than appear on T cells (29,79). Recently, Jensen et al. (80) have shown that, like T cells (26), activated B cells down-regulate CD45RA and shift to the expression of CD45RO.

CD45 expression has been most extensively studied on T cells. Among peripheral blood T cells, CD45RA and CD45R0 are reciprocally expressed, CD45RA$^+$ cells accounting for 60% of the total, and CD45R0 appearing on the remaining 40% (11). These numbers do not reflect the differences between the major T cell subsets, however, since 70-85% of CD8$^+$ cells express high molecular weight forms of CD45, whereas closer to 50% of CD4$^+$ cells are CD45RA$^+$ (11,81,82). The question of whether or not individual resting T cells normally express more than one isoform is still unresolved, although it is known that some cell lines express more than one species of CD45 mRNA (83). In these cell lines one type of mRNA predominates and it is not known whether the others are translated. Resolution of this question awaits the development of antibodies which specifically recognize additional restricted CD45 epitopes.

The proportions of CD45RA$^+$ and CD45R0$^+$ cells among peripheral blood T cells of certain patient populations differs significantly from that of normal individuals, in ways that support the concept of CD45R0$^+$ subset as memory cells. For example, the CD45RA$^+$ set is selectively decreased in multiple sclerosis (84), multiple myeloma (82), rheumatoid arthritis (85), and in allergic individuals (86). The percentage of CD45RA$^+$ cells returns toward normal values in allergic patients undergoing hyposensitization therapy (86).

The majority of thymocytes are exclusively CD45R0$^+$ (17). It has been reported that only a few cells within the thymus express higher molecular weight forms of CD45 (17), and that these are mostly B cells and a small minority of medullary thymocytes with mature "single-positive" phenotypes (14,16,22,87). Recently, however, an extensive FACS analysis of thymus subsets using two- and three-color staining procedures, has revealed a significant proportion (10 to 30%) of CD45RA$^+$ thymocytes (9), although the density of expression is low on the majority of cells. CD45RA$^+$ cells are found within all of the thymic subsets defined by CD3, CD4 and CD8 (9) and the majority coexpress CD45R0 (9). CD45RA$^+$ cells are found with greatest frequency among the immature CD3$^-$4$^-$8$^-$ progenitor set (about 80%) and in the mature CD3$^+$4$^+$ and/or 8$^+$ "single positive" subsets (50%) (9). Immunohistological staining of infant human thymus shows that CD45RA$^+$ cells are primarily located in the medullary region. Cortical areas, as defined by CD1 positivity, are predominantly CD45RO$^+$ with a few scattered CD45RA$^+$ cells (9,16,87).

Functional Studies: Antibodies to both common and restricted determinants on CD45 have been found to have inhibitory effects in a variety of assays of cellular function (29). B cell proliferation induced by either anti-IgM (88), lipopolysaccharide (89), or anti-CDw40 (90) is inhibited by both types of anti-CD45 antibodies. Antibodies to CD45 common determinants inhibit NK cytolysis (29,91,92). In addition, NK cell experiments provide the only demonstration to date, of involvement of CD45 in cell-cell interactions (93). In these studies, purified CD45 incorporated into liposomes inhibited conjugate formation between NK cells and their targets (93). Inhibition was blocked if the liposomes were pretreated with anti-CD45 or with endo-b-galactosidase, consistent with the finding that carbohydrate structures on CD45 are involved in NK target binding (93). Inhibition of CTL cytolysis in the presence of antibodies against CD45 has been observed by some workers (29,94) but not by others (29), possibly reflecting differences in antibody specificities (29).

As discussed earlier, CD45 expression is developmentally regulated on T cells. Work described in this thesis includes the first experiments exploring the role of CD45 in thymocytes. However, there are a number of studies on mature T cells, both on the effects of antibodies against CD45, and on differential functional capacities of T cell subsets defined by CD45 antibodies.

There are several reports of CD45 antibodies affecting *in vitro* proliferation of mature T cells. The anti-CD45RA antibody WR16 inhibits pokeweed mitogen-induced T cell proliferation (95), and antibodies against CD45 common determinants inhibit PHA-induced proliferation of both human (96,97) and mouse (98) T cells. In one of these studies the inhibition was shown to be accompanied by reduced IL-2 production and IL-2 receptor expression (96). In other studies, anti-CD45 antibodies have augmented proliferation induced by suboptimal mitogenic combinations of anti-CD2 antibodies (97), and replaced the requirement for monocytes in the stimulation of purified populations of T cells by anti-CD3 antibodies coupled to sepharose beads (94). Anti-CD45RA antibodies have augmented proliferation induced by suboptimal doses of PHA (26,98) and also by anti-CD2 and anti-CD3, but, in the latter case, only when IL-2 is present in excess (26). However, Fab' fragments of anti-CD45RA blocked proliferation, indicating a requirement for cross-linking of CD45RA molecules (26). The enhancement of proliferation was apparently due to an increase in the numbers of cells induced to express IL-2 receptors (26). Signal transduction through CD45RA was thus functionally linked to the IL-2/IL-2R system.

In a recent study, Ledbetter et al (99) showed that proliferation induced by immobilized anti-CD3 was inhibited by either CD45RA mAb or an antibody against a CD45 common determinant, but only if they too are immobilized. Similarly, soluble anti-CD45 enhanced proliferation induced by CD2 or CD28 antibodies, but inhibited proliferation if cross-linked to the CD2 or CD28 mAb using an anti-mouse kappa chain antibody (99). An early measure of cellular activation is a rise in intracellular calcium $[Ca^{2+}]_i$ (100). Interestingly, the increase in $[Ca^{2+}]_i$ induced by CD3, CD2 or CD28 antibodies (101-103) is inhibited by anti-CD45 when the two are cross-linked in a biotin/ avidin system, however the Ca^{2+} response induced by anti-CD4 is greatly enhanced (99). Thus it appears that anti-CD45 has different modulating effects on T cell activation, depending on the molecules with which it associates.

In summary, it is clear that the CD45 molecule can transduce signals to the interior of cells, but the precise nature of the signals, and their relevance to normal cellular function is unknown. The pattern of response to CD45 antibodies is complex, particularly among T cells, and presumably reflects differences in assay conditions and activation states of the cells.

A second approach to studying the role of CD45 has been the comparison of properties held by peripheral blood T cell populations which are either CD45RA$^+$ or CD45R0$^+$ (22,95,104). Using the antibodies 2H4 (anti-CD45RA) and 4B4 (an antibody against CD29, the expression of which largely overlaps with the CD45R0$^+$ subset) it was shown that CD4$^+$CD45RA$^+$ cells did not respond to recall antigens, but did proliferate in AMLR and to polyclonal stimulators, and exhibited suppressor-inducer function in a pokeweed mitogen-driven system of B cell activation (104). In contrast, the reciprocal population of CD4$^+$ 4B4$^+$ cells responded well to recall antigens, and provided help to B cells (22). Thus, the 2 subsets could be assigned separate functions and were thought to belong to separate lineages. Recent evidence, based on differential lymphokine production, suggests that similar subsets in the mouse may correspond to the Th1 and Th2 sets of T cell clones (73). However, this does not appear to be the case in humans (105). It remains to be determined whether this is due to a fundamental difference between the murine and human CD45 systems, or to the fact that the specificity of the antibody used in the murine study is dissimilar to anti-CD45RA antibodies used in human studies. Nonetheless, the human CD4 subsets do differ with respect to lymphokines produced: the CD45R0$^+$ set exclusively produces interferon-γ (105-108) and IL-4 (105,109), and, while both subsets produce IL-2, there is a difference in the kinetics of IL-2 production (106,108-111).

There are contradictory data on the differential activation properties of the CD45RA$^+$ and CD45R0$^+$ subsets. For example, mitogenic anti-CD2 antibodies have been shown to induce proliferation of only CD45R0$^+$ cells in some studies (107,112), but only CD45RA$^+$ cells in others (113). One group was unable to find any difference between the 2 subsets in their responses to anti-CD3-induced proliferation (113,114), whereas others find that CD45R0$^+$ cells are more responsive to anti-CD3 (28,115). Salmon et al. (109) and Dohlsten et al.(108) have found that CD45RA$^+$ cells proliferate more strongly to mitogens than do CD45R0$^+$ cells, while Byrne et al. report no difference (28).

The reasons for these contradictory results are not obvious. Some differences between donors have been reported (28), and certainly there are differences in cell fractionation methods. However, some workers have taken great care to confirm their results using cell populations prepared by different methods; in particular, by both positive and negative selection procedures (28). Despite the confusion of experimental results, the point can be made that there are profound differences in T cells which are positive for the higher m. w. forms of CD45 versus those that are CD45R0$^+$. The latter are almost certainly antigen-experienced memory cells, and it is thus plausible that they would be more easily activated than their CD45RA$^+$ precursors, particularly by stimuli received via the antigen-receptor. Conversely, CD45RA$^+$ cells may require additional signals for complete activation, possibly through the alternate CD2 pathway, or even through CD45RA itself. The question remains: is the presence or absence of CD45RA molecules a crucial feature of the functional differences between cell types? Given that CD45 is a tyrosine phosphatase and transduces either positive or negative signals when stimulated by antibodies, it is likely that it serves some regulatory and/ or accessory function. A major advance will be made when the putative ligands for the variable external domains are identified.

Data presented in this thesis are derived from investigations prompted by predictions of the hypothesis outlined at the beginning of this chapter. In brief, the hypothesis states that CD45RA marks the generative thymic lineage and is essential for the survival of thymocytes. The data is presented in 3 parts. First, an analysis and comparison of the kinetics of CD45 mRNA transitions in PBMC and thymocytes was done, on the premise that, in contrast to PBMC, the shift from high to low m.w. CD45 isoforms would not occur in thymocytes following stimulation, unless such stimulation initiated a programmed cell death. These initial experiments raised interesting questions regarding the properties of CD45 mRNA, prompting further studies in this area. All of the work on CD45 mRNA is detailed in Chapter 3.

Chapter 4 describes work which demonstrates that CD45RA molecules transmit stimulatory signals to immature thymocytes, and Chapter 5 provides evidence that CD45R0-depleted $CD3^-4^-8^-$ thymocytes are an even more immature population of cells than undepleted $CD3^-4^-8^-$ cells, as predicted by the hypothesis.

Chapter 6 details a study of the activation properties of $CD3^-4^-8^-$ thymocytes. In these experiments, proliferation assays were used to investigate the effects of stimulating cells via CD45 as well as other T cell-surface signaling molecules, CD2 and CD28 in particular. In addition, the differential ability of antibodies against common and restricted determinants to modify signals initiated through CD2 and CD28 was examined.

II. MATERIALS AND METHODS

Tissue samples: Peripheral blood mononuclear cells (PBMC) were obtained from buffy coat preparations from healthy donors to either the Melbourne Red Cross Blood Bank, or the Canadian Red Cross Blood Transfusion Service, Edmonton. Fragments of human thymus were obtained as part of the routine surgical procedure, from children aged 1 week to 13 years undergoing cardiac surgery either at the Royal Childrens Hospital, Parkville, Victoria, Australia, or at the University of Alberta Hospital, Edmonton. Placental tissue was obtained from the Department of Obstetrics, University of Alberta Hospital. Fragments of human spleen were obtained from a patient undergoing splenectomy at the University of Alberta Hospital, Edmonton.

Cell preparations: *Peripheral blood mononuclear cells (PBMC)*: Buffy coat cells were diluted two times in cold RPMI and centrifuged over Ficoll-Paque. PBMC collected from the interface were washed three times in cold RPMI. In some experiments PBMC were depleted for CD45R0$^+$ cells and for B cells, using the mAb UCHL1 and B4 in the modified procedure described below for thymocyte subsets. Phenotypes of unfractionated PBMC and UCHL1/B4-depleted PBMC is shown for a representative sample in Table 2.1.

Unfractionated thymocytes: Thymus tissue was initially chopped in sterile RPMI containing 10% fetal calf serum (RPMI/FCS) and DNase, and then passed through a sieve to obtain a single cell suspension. Cells were washed, and red and dead cells removed by centrifugation over Ficoll-Paque, followed by two washes in cold RPMI.

Thymocyte subsets: The majority of experiments involved the use of CD3$^-$4$^-$8$^-$ thymocytes. In some experiments this subset was also depleted for CD45R0$^+$

TABLE 2.1

Phenotypes of unfractionated and CD45R0⁻, B⁻ PBMC

	Unfractionated	CD45R0⁻ B⁻
CD4	41.5[a]	44.0
CD8	18.6	30.0
CD19 (B cells)	29.0	2.6
CD45RA	61.0	94.0
CD45R0	45.0	7.8
GαM Ig-FITC	1.4	13.0

[a] % positive cells

Phenotypes were determined by either single direct IF using mAbs OKT4 (CD4), OKT8 (CD8), FMC63 (CD19) and BT25 (CD45RA), or single indirect IF using UCHL1 (CD45R0). Depletions were done using UCHL1 and FMC63. For single IF, electronic gates were set for analysis based on staining with isotype-matched control antibodies. For indirect IF, control staining with GαM Ig always shows higher values in depleted populations as compared to unfractionated cells. Results shown are representative of 4 experiments. Data by Eva Pruski.

cells. One experiment was performed with unfractionated thymocytes depleted only for CD45R0$^+$ cells. These subsets were prepared from unfractionated thymocytes as follows: up to 3 x 10^9 thymus cells were suspended in monoclonal antibody supernatants (OKT3, OKT4, OKT8, FMC63, and UCHL1 where indicated) for 60 min on ice. FMC63 (CD19) was used to deplete B cells which constitute up to 1% of unfractionated thymocytes (116,117), and are otherwise enriched in populations depleted of CD3$^+$, CD4$^+$ and CD8$^+$ cells. Absorbed rabbit serum was then added as a source of complement, to 20% final concentration. The volume was made up to 50 ml with pre-warmed RPMI and incubated at 37°C for 40 min. DNase (0.1%) was then added and the cells pelleted by centrifugation, resuspended in fresh medium, centrifuged over Ficoll-Paque, and washed twice. The resulting cell suspension was 99% viable. Cells were further depleted of antibody binding cells by two rounds of incubation with magnetic Dynabeads (0.2 ml Dynabeads/10^7 cells) coupled to anti-mouse Ig (DYNAL, Oslo, Norway) for 20 min on ice. Cells with bound Dynabeads were removed with a magnet. The resulting cell suspension had from 2-5% cells with a low density of mouse Ig on their surface, and by immunofluorescence (IF) analysis, from 1-5% cells expressing a very low density of the markers used for depletion. In later experiments the complement step was omitted and correspondingly greater amounts of Dynabeads, in four depletion steps, were used. This modification resulted in more consistent yields, even lower levels of contamination by unwanted cell types, as well as in cell populations which more accurately reflected the phenotypes expected from FACS analysis on populations electronically gated to include only the CD3$^-$4$^-$8$^-$B$^-$ set of thymocytes. Phenotypes of the various thymocyte subsets are given in Tables 4.1 and 4.2 in Chapter 4.

Monoclonal antibodies: All antibodies and antibody conjugates used in this work are listed in Table 2.2, along with their sources and,where applicable, references which document their specificities and methods of production.

Immunofluorescence: Purified thymocytes or PBMC were stained by incubating from 1 x 10^5 to 5 x 10^5 cells with the relevant antibodies, for 30 min at 4^oC, in V-bottom microtiter wells. For indirect staining the cells were then washed twice with PBS containing 0.05% BSA, and incubated again for 30 min at 4^oC, with a fluorochrome (FITC or PE) -conjugated second antibody. After staining and washing, cells were fixed in 1% formalin and analyzed by flow cytometry using a modified FACS II or FACSCAN (Becton Dickinson, Mountain View, Calif.). For three color analysis, a separately aimed and focussed Argon laser emitting in the UV range was used to activate blue fluorescence from coumarin (blue), and an Argon laser exciting at 488 nm to detect fluorescein (FITC, green) and phycoerythrin (PR, red). Dead and red cells were excluded electronically on the basis of forward light scatter. Each experiment included staining with isotype matched control antibodies to establish the specificity of antibody binding. For those antibodies detected with indirect IF, controls to detect binding of the fluorochrome-coupled second stage reagent were always included; both avidin and goat anti-mouse Ig were used. In the absence of clear-cut peaks, IF was considered to be negative in channels 0-50, dim in channels 51-70, bright in channels 71-149, and very bright in channels 150-256.

Cell Cultures: Cells were cultured at 10^6/ml in RPMI/10% FCS with 50 µg/ml gentamycin (GIBCO), and 50 U/ml penicillin, 50 µg/ml streptomycin (GIBCO), and incubated at 37^oC in 5% CO_2/air. Other culture conditions varied between different types of experiments, and the details are provided with the results. In general, when the

TABLE 2.2

Antibodies

Antibody	Specificity	Isotype	Species	Source	Reference
OKT4	CD4	IgG2b	Mouse	ATCC[a]	
OKT8	CD8	IgG2a	Mouse	ATCC	
OKT3	CD3	IgG2a	Mouse	ATCC	
T11-FITC	CD2	IgG1-κ	Mouse	Coulter[b]	
T3-FITC	CD3	IgG1-κ	Mouse	Coulter	
B4-FITC	CD19	IgG1-κ	Mouse	Coulter	
Leu2-FITC	CD8	IgG2a	Mouse	BD[c]	
Leu3-FITC	CD4	IgG1	Mouse	BD	
Leu2-PE	CD8	IgG2a	Mouse	BD	
Leu3-PE	CD4	IgG1	Mouse	BD	
Hle1-FITC	CD45	IgG1-κ	Mouse	BD	
UCHL1	CD45R0	IgG2a	Mouse	Dr. Peter Beverley[d]	19
FMC44	CD45RA	IgG1	Mouse	Dr. Heddy Zola[e]	9
BT25	CD45RA	IgG1	Mouse	"	9
FMC63	CD19		Mouse	"	9
ASH1621	CD45	IgG2a	Mouse	Dr. Ian McKenzie[f]	9
6F10.3	CD2	IgG1	Mouse	Dr.Claude Mawas[g]	118
39C1.5	CD2	IgG2a	Rat	"	118
9.1	CD2	IgG3		Dr. Bo Dupont[h]	119
3AC5	CD45RA	IgG2a	Mouse	Dr. Jeff Ledbetter[i]	120
9.4	CD45	IgG2a	Mouse	"	121
9.6	CD2	IgG2b	Mouse	"	122
9.3	CD28	IgG2a	Mouse	"	123
G19.4	CD3	IgG1	Mouse	"	120
187.1	mouse κ	IgG1	Rat	"	124
Homoconjugates					
3AC5/3AC5				Dr. Jeff Ledbetter	
9.4/9.4				"	
9.6/9.6				"	101
9.3/9.3				"	101
G19.4/G19.4				"	102
Heteroconjugates					
9.6/3AC5				Dr. Jeff Ledbetter	
9.3/3AC5				"	
9.6/9.4				"	
9.3/9.4				"	
9.4/3AC5				"	
9.4/G19.4				"	99
3AC5/G19.4				"	

[a] American Type Culture Collection, Rockville,MD; [b] Hialiah,FL; [c] Becton-Dickinson Mountain View, CA;[d] University College, London; [e] Flinders Medical Center, Australia; [f] University of Melbourne, Australia; [g] INSERM U.119, Marseille;[h] Sloan-Kettering Inst., New York; [i] Oncogen, Seattle,WA.

measurement of IL-2 mRNA was to be performed, thymocytes were cultured for 24 h. PHA (GIBCO), PMA (Sigma Chemical Co., St. Louis, MO), Ionomycin (Behring Diagnostics, La Jolla, CA), and mAb were added in various concentrations and combinations as indicated in the text and figure legends. In some of the early experiments cultures were supplemented with 10% T cell growth factors (TCGF) derived from irradiated PBL stimulated with 2 μg/ml ConA for 3 days. For studies on the induction and degradation kinetics of CD45 mRNA, the following reagents were employed: Actinomycin D 5 μg/ml (Sigma), cycloheximide 20 μg/ml (Sigma), cyclosporin 200 ng/ml (Sandoz Canada Inc., Dorval, Quebec) and various mAb, as indicated in the text and figure legends.

Proliferation assays: Thymocytes were cultured at 10^6/ml in RPMI/10% FCS with 50 μg/ml gentamycin (GIBCO), and 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO), in 96-well flat-bottomed plates, in 200 μl volumes, for 4-5 days at 37°C in 10% CO_2/air. In later experiments, as indicated in the text, 1% human AB serum (Canadian Red Cross Blood Transfusion Service, Edmonton, Alberta) was added as a source of human transferrin (125). PMA (Sigma), recombinant human IL-2 (Dr. Vern Paetkau, Dept. of Biochemistry, University of Alberta), recombinant murine IL-7 (Immunex, Seattle, WA) and various combinations of mAb were added as indicated. One μCi/well of ^{3}H-thymidine (NEN Research Products) was added for the last 16 h of the culture, and the cells were harvested onto filter paper (Flow Laboratories) using a Skatron A.S. semi-automated cell-harvester (Flow Laboratories). Samples were counted for 30 seconds in a LKB 1218 Rackbeta liquid scintillation counter, and results are expressed in cpm. All tests were done either in triplicate or in quadruplicate and the standard error of the mean was generally <10%.

Proliferation assays using peripheral blood mononuclear cells were performed as above, except that the cells were cultured at 5 x 10^5/ml for 3 days, and ^{3}H-thymidine was added 6 h before harvesting.

RNA preparation and Northern analysis: In most experiments, with the exceptions described below, total cytoplasmic RNA was prepared using a rapid method described by Pearse and Wu (126). This method is ideal for preparing RNA from small numbers of cells and also for processing many samples simultaneously. Cells were washed in PBS then resuspended, with vigorous vortexing, in cold lysis buffer (150 mM NaCl; 10 mM Tris-HCl, pH 8.0; 2 mM MgCl; 0.5% NP-40 (Sigma); 10 mM vanadyl ribonucleoside complexes (Biolabs)). Up to 5 x 10^6 cells were resuspended in 150 µl of lysis buffer. After vortexing, the nuclei were removed from the lysate by centrifugation for 7 min at 2000 x *g*. An equal volume of protein denaturing buffer (7 M urea, 450 mM NaCl, 10 mM EDTA, 1%SDS, 10 mM Tris-HCl, pH 7.4) was mixed with the supernatant and proteins were removed by extracting once with phenol/chloroform. Samples were stored for later analysis in 2-3 volumes of 95% ethanol at -70^{o}C.

When RNA was prepared from large numbers of cells, for example from the Raji and HL60 cell lines, and from the human spleen cells used in experiments described in chapter 3, the method of Chirgwin et al. was used (127,128). Up to 5 x 10^7 cells were washed once in PBS and resuspended in 3 ml of freshly-prepared 4 M guanidinium thiocyanate solution containing 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate pH7.0 and 0.1 M 2-mercaptoethanol. The suspension was homogenized using a Polytron (Brinkman) tissue homogenizer, layered over 2 ml of filtered 5.7 M cesium chloride containing 0.1 M EDTA, in a tube suitable for the Beckman SW50.1 rotor, and centrifuged for 12-20 h at 36,000 rpm at 20°C. The supernatant was removed using a suction flask and the RNA pellet rinsed briefly with about 300 µl of cold water before resuspending in about 200 µl of 0.1% SDS/25 mM EDTA. The RNA was then ethanol-precipitated, resuspended in 0.1% SDS/25 mM EDTA and the concentration estimated by measuring ethidium bromide fluorescence at pH 8 in a Gilson Spectra/Glo Fluorometer (129).

Ethanol-precipitated RNA was resuspended in 10 µl of 1X MEN buffer containing 50% formamide and 2 M formaldehyde, heated to 65°C for 5 min, and either fractionated in 0.8% agarose gels containing 0.66 M formaldehyde before transferring to nylon filters (Hybond-N, Amersham), or bound directly to the filters using a Schleicher and Schuell "dot-blot" apparatus. The RNA was cross-linked to the filters by exposure to 312 nm light for 5 minutes using a UV transilluminator. Prehybridization was performed for at least 2 h at 42°C in 50% formamide, 3X SSC, 1X Denhardt's solution, 0.3 µg/ml sheared salmon sperm DNA. Filters were hybridized overnight at 42°C in the same buffer containing 1-2 x 10^6 cpm of labelled probe. The filters were washed first in 2X SSC, 0.1% SDS at 42°C for 15 min, then in 1X SSC, 0.1% SDS at 65°C for 30 min, and finally in 0.1% SDS at 22°C for 15 min. Autoradiography was performed at -70°C using Kodak XAR-film with intensifying screens, for times indicated in the figure legends.

DNA Preparation and Southern analysis: A method described by Williams (130) was used to prepare DNA from the small numbers of cells obtained from thymus tissue after depletion with various combinations of antibodies. This method also lends itself well to batch analysis, as several samples can be easily processed simultaneously. Up to 2 x 10^6 cells were washed once in PBS in an eppendorf tube, and the dry pellet stored at -70°C for later analysis. The pellet was thawed into 10 µl of buffer A (0.5% sodium lauroyl sarkosinate, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0), vortexed, and warmed to 50°C. Next, 10ml of buffer A containing 2% LGT agarose (Pharmacia) and 0.5 µg/ml of freshly added proteinase K at 50°C was added, and incubated for 2 h at 50°C. The mixture was allowed to solidify for 5 min on ice, and then washed at 4°C with 3 changes of TE over the next 24 h, to remove the detergent. The pellet was melted by heating to 65°C for 5 min, then transferred to 37°C before adding 3.5 µl of 10X restriction enzyme buffer, 1 µl nuclease-free BSA (5 µg/ ml) and sufficient water to bring the volume to 35 µl after addition of the enzymes. One µl of 10 µg/ml DNAse-free RNAse was added and incubated for 15 min at 37°C, then 30 units of restriction enzyme was added and incubated overnight at 37°C. For Southern analysis, digested samples were heated to 65°C and 5 µl of loading solution (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) were added prior to loading onto a 0.7% agarose gel in a Mini-Sub (Bio-Rad) apparatus.

Often this method was combined with the rapid RNA preparation method described above, to obtain both DNA and RNA from the same cells (126). When this was done the pellet of nuclei obtained after centrifugation of the cells in lysis buffer (see "RNA preparation" above) was saved and treated exactly as described above for whole cells.

Placenta and thymus tissue fragments were used as sources of germ-line and rearranged TCR genomic DNA, respectively. About 10 g of freshly dissected tissue was dounce homogenized in 60 ml 5% citric acid and filtered through 10 layers of sterile gauze. The filtrate was divided into 2 tubes and centrifuged for 5 min at 2500 x *g* (128). Each pellet was suspended in 10 ml of 5% citric acid, layered over 15 ml of 0.88 M sucrose in 5% citric acid in a 30 ml Corex tube, and centrifuged for 5 min at 5000 x *g*. The pelleted nuclei were then washed twice in 10 mM Tris, pH 7.4, containing 10 mM NaCl and 25 mM EDTA, and resuspended in 10 ml of the same buffer. SDS was added to 1% final concentration, then proteinase K was added to 1mg/ml and the mixture incubated for 2 h at 37°C. The proteinase K step was repeated, to ensure completed digestion of proteins, before adding 1 ml of 5 M NaCl and extracting once with phenol/chloroform. The DNA was extracted from the lower aqueous phase by ethanol precipitation, and then resuspended and stored in TE buffer at 4°C.

Southern gels were run overnight at 25 V, then subjected to partial acid depurination by soaking in 0.25 M HCl for 10-20 min before transferring to Zeta-probe nylon membranes (Bio-Rad) with 0.4 M NaOH. Prior to prehybridization the filters were washed 2-3 times in 0.1X SSC, 0.1% SDS at 42°C to remove any remaining traces of alkali. Prehybridization was performed at 42°C for at least 2 h, in 50% formamide, 5X SSPE, 0.1% SDS, 5X Denhardts, 200 μg/ml heat-denatured salmon sperm DNA. Hybridization was performed overnight at 42°C, in 50% formamide, 5X SSPE, 0.1% SDS, 1X Denhardts, 10% dextran, 200 μg/ml heat-denatured salmon sperm DNA, 0.34% non-fat dried milk powder and 1-2 x 10^6 cpm of labelled probe. Filters were rinsed first in 2X SSC, 0.1% SDS at 22°C for 10 min, then at 65°C for 30 min in 0.5X SSC, 0.1% SDS. Autoradiography was performed at 70°C using Kodak XAR-5 film with intensifying screens, for times indicated in the figure legends.

Probes: All cDNA probes used in this work are listed in Table 2.3. The strategy for making the probes specific for the variable exons of CD45 was as follows. The pHLC-2 cDNA, representing ≅1kb of the 5' end of CD45 mRNA (44), was received in the pcD cloning vector from Dr. Ian Trowbridge. The insert was purified by first cutting it out of of the vector with *Bam* H1, and then separating it from the vector by

TABLE 2.3

cDNA probes

cDNA	Size[a]	Source	RE fragment	Size[a]	Region recognized	Ref
pY1.4	1200	Dr. T. Mak	-	-	V, D, J and C sequences of human TCR α	131
JUR-β2	770	Dr. T.Mak	-	-	J and C regions of human TCR β	132,133
HGP02		Dr. T.Mak	-	-	V, J and Cγ1 regions of human TCR γ	134
CD3 δ	800	Dr. J.P.Allison	-	-	CD3δ coding region plus 5' and 3' UT sequences	135
Actin		Dr. V. Paetkau	-	-		136
IL-2	820	Dr. V. Paetkau	*Stu* I	540	coding region of IL-2	137
IL-2R	1300	Dr. V. Paetkau	*Sac* I/*Xba* I	737	coding region of IL-2R	138,139
pHLC-1	3600	Dr. I. Trowbridge	*Bam* HI	750	250 aa in the cytoplasmic region of CD45,proximal to the plasma membrane	44
pHLC-2	1000	Dr. I. Trowbridge	*Sfa* N1	466	155 aa of the 161 aa CD45 variable region insert	44

[a] number of base pairs

electophoresis in a cold 1% low melting point agarose gel. Gel slices containing the relevant bands were cut out, melted at 65^{o}C, and phenol extracted. Two *Sfa* N1 restriction sites were found to be present in the pHLC-2 sequence, one close to the 5' end of exon 4 and the other close to the 3' end of exon 6. Digestion with this enzyme therefore yielded a 466 bp fragment which included almost all of the sequence encompassing the 3 variable exons, and no extraneous sequence. Five µg of purified pHLC-2 was digested with *Sfa* N1 and electrophoresed in 3 lanes of a 3.5% polyacrlamide gel. The 466 bp fragment was purified from the gel slices and, since *Sfa* N1 is an asymmetric cutter, the ends were filled in with DNA polymerase (140, pg113) prior to blunt-end ligation into the vector pGEM 3Z, previously prepared by digestion with *Hinc* 2 and dephosphorylation (140, pg 133). The mixture was then used to transform competent cells, and 24 colonies were screened for the presence of the appropriate fragment. One was selected and expanded, and the subcloned *Sfa* N1 fragment was isolated and purified after its sequence was checked by restriction mapping.

Probes were labelled by random priming using Amersham's Multiprime DNA labelling system to a specific activity of $\cong 10^9$ cpm/µg.

III. KINETICS OF TRANSITION FROM HIGH TO LOW MOLECULAR WEIGHT ISOFORMS OF CD45 FOLLOWING ACTIVATION OF T CELLS AND THYMOCYTES

Phenotypic changes in the composition of T cell surface membrane proteins appear to be fundamental to the process of maturation in response to antigenic stimulation. The pattern of antigenic determinants detectable on the cell surface allows tentative definition of that cell as a naive Ag-inexperienced T cell or an Ag-experienced memory T cell. CD45 antigens are particularly useful in this regard. Upon stimulation with mitogen or Ag *in vitro*, PBMC lose their CD45RA and acquire CD45R0. This process takes from 2-4 days and is characterized by co-expression of both markers. During the transitional period the density of CD45RA gradually decreases as the density of CD45R0 increases, although the total density of CD45, as measured by the expression of determinants common to all isoforms, is considerably higher at later stages of culture than at initiation of culture (14). This indicates a net gain in surface CD45 concomitant with the transition from CD45RA to CD45R0. The transition in expression of CD45 isoforms appears to be an irreversible unidirectional process. Purified CD45R0$^+$ cells do not revert to expression of CD45RA (20). Transitional changes from CD45RA to expression of CD45R0 do not reflect selective overgrowth of antigen-inexperienced T cells in these cultures. Limiting dilution cloning of purified CD45RA$^+$ T cells unequivocally demonstrated that all CD45RA$^+$ progenitors give rise exclusively to CD29$^+$ progeny (14). CD29 is the β chain of the VLA family of integrin molecules, its expression largely overlapping with CD45R0 in peripheral blood T cells. Historically, the CD29 mAb 4B4 has been used to define the reciprocal subset to CD45RA, due to the paucity of mAb that specifically recognizes CD45R0.

Although the transition from CD45RA to expression of CD45R0 occurs over a 2-4 day period, it is not clear whether this reflects a slow turnover of glycoprotein on the cell surface after rapid changes in patterns of mRNA splicing, or whether alternative splicing is initiated at 2 to 4 days post-stimulation. Therefore, in this study the cell surface phenotype was correlated with CD45 mRNA synthesis as a function of time after stimulation of PBMC with PHA.

RESULTS AND DISCUSSION

PBMC lose CD45RA and gain both CD45R0 and CD29 after stimulation. In order to map the phenotypic changes occurring after stimulation with PHA, PBMC were cultured for 7 days and the surface phenotype analyzed by IF at multiple sequential time points. For simplicity, only data from days 0, 1, 3 and 7 are shown in Figure 3.1. As previously reported (14) the density of CD45 common determinants increases roughly 3-fold over 7 days of culture. The whole PBMC population exhibits increased CD45 within the first 24 h of culture (Fig. 3.1., *column 1. Vertical dashed lines* represent the peak fluorescence channel of unstimulated PBMC). CD45RA, expressed on 60% of PBMC at day 0, increases on days 1 and 2 as reported by others (20,141), but falls to nearly undetectable levels between days 3 and 4 and remains low at day 7, confirming other reports (14,20,25,26). CD45R0 is found on 40% of PBMC initially and shows increased density by 24 h, continuing to increase in density and number of positive cells until day 7, when 90% of cells express very high density of CD45R0. A similar pattern is seen for CD29 which initially is present at high density on 40% of PBL. By 24 h the low density CD29$^+$ PBMC have become high density and by day 3 to 7 100% of cells are CD29$^+$. By day 3 of culture nearly all cells express CD3 (see legend, Fig.3.1), defining them as T cells, and the CD3 increases in density with time in culture.

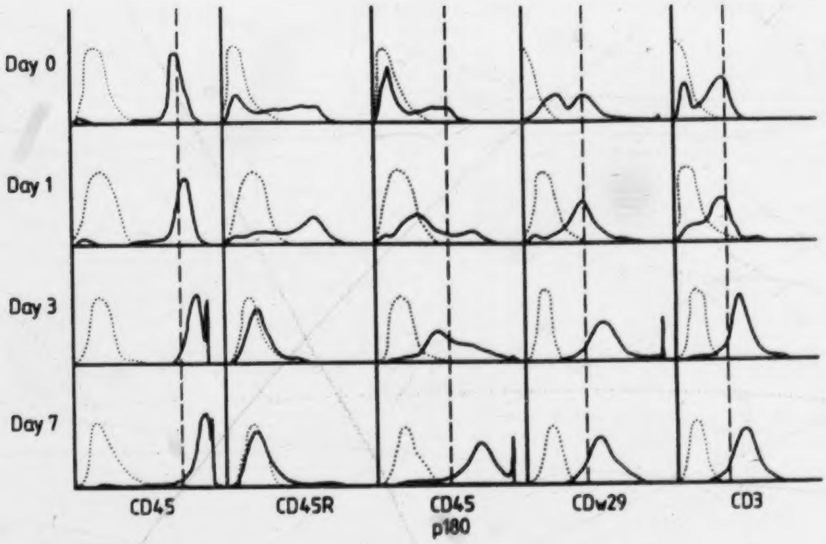


Figure 3.1. Phenotypic analysis of PHA-stimulated PBMC: loss of CD45RA and acquisition of CD45R0 and CD29. CD45 was detected by ASH1621, CD45RA by FMC44, CD45R0 by UCHL1, CD29 by 4B4 and CD3 by T3-FITC. *Dotted lines* represent staining in the negative control. *Dashed lines* indicate the peak IF channel for resting PBMC. Cultures were phenotyped at days 0, 1, 2, 3, 5 and 7 but only the time points above have been reported for simplicity. The density of CD2 also increased over time in these cultures. At day 0, 70% of PBMC were CD2$^+$ CD3$^+$ T cells. By day 3, 90% were T cells and 100% by day 7.

Transitions in CD45 mRNA in mitogen-stimulated PBMC. The cell surface changes detailed above could result from either a slow membrane turnover of CD45RA after immediate switching of mRNA splicing, or from a delayed activation of the alternative mRNA splicing pattern required to produce mRNA encoding CD45R0. To resolve this, CD45 mRNA from PBMC was analyzed at various time points after stimulation with PHA (Fig. 3.2), using a probe recognizing sequences in the cytoplasmic domain of CD45 (44). Since the 5.4 and 4.8 kb mRNA species of CD45 are sometimes difficult to resolve, poly(A+) RNA from cell lines expressing only the 5.4 kb or the 4.8 kb mRNA were used as control markers on Northern gels (44,45).

Figure 3.2A shows the lack of CD45 mRNA in a hepatocyte cell line, 5.4 kb mRNA from Raji cells, and 4.8 kb mRNA from HL60 cells. Thymocytes, which are predominantly CD45R0^{+} (9,10,19), express the 4.8 kb mRNA (Fig. 3.2A). Two different experiments are shown in Figure 3.2, A and B. The copy number for CD45 mRNA was apparently low in resting PBMC, because it was only detectable in some PBMC preparations and only when RNA was prepared from higher numbers of cells as shown in Figure 3.2B. The predominant mRNA is 5.4 kb but a smaller amount of 4.8 kb message is detectable; these bands are seen if RNA from 4 x 10^{6} but not 2 x 10^{6} cells are loaded on the gel. Figure 3.2B is included to show the amount of total CD45 mRNA synthesized after stimulation. Within 24 h of stimulation the 5.4 kb mRNA has disappeared and only 4.8 kb mRNA is detectable. The amount of 4.8 kb mRNA reaches its highest level by day 3 and decreases after that. At no point is 5.4 kb mRNA detectable in stimulated PBMC. Figure 3.2A shows lanes loaded with equivalent amounts of RNA from resting and stimulated cells. This reduction in the amount of mRNA loaded permits clearer definition of the size of the CD45 mRNA. No CD45 mRNA is detectable from resting PBMC and by 24 h post-stimulation only the 4.8 kb

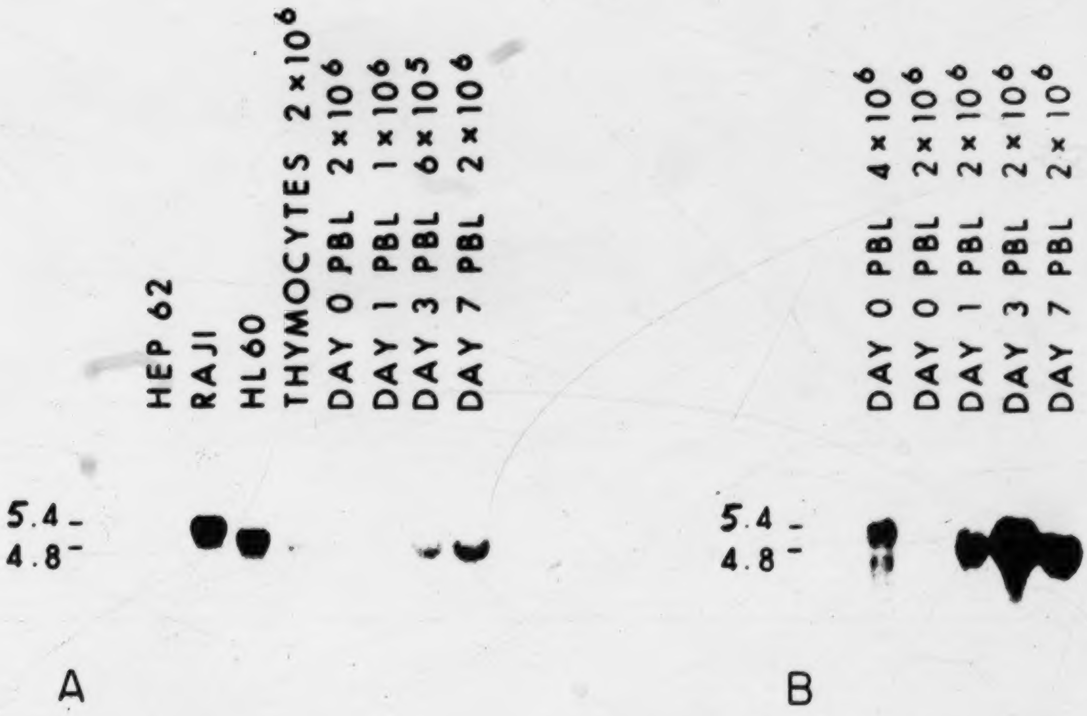

HEP 62
RAJI
HL60
THYMOCYTES 2×10^6
DAY 0 PBL 2×10^6
DAY 1 PBL 1×10^6
DAY 3 PBL 6×10^5
DAY 7 PBL 2×10^6
5.4
4.8
A
DAY 0 PBL 4×10^6
DAY 0 PBL 2×10^6
DAY 1 PBL 2×10^6
DAY 3 PBL 2×10^6
DAY 7 PBL 2×10^6
5.4
4.8
B

Figure 3.2. CD45 mRNA species in resting and activated PBMC. RNA was prepared from the indicated number and type of cells and analyzed on Northern gels by probing with the cDNA insert from pHLC-1 (44). In *A*, approximately equivalent amounts of RNA were loaded in each lane as assessed by the relative intensities of the ribosomal RNA bands on ethidium bromide staining. Poly(A+) RNA from HepG2, Raji and HL60 was loaded at 2 μg/lane. In *B*, RNA from equivalent cell numbers was loaded. *A* and *B* represent separate experiments. Both *A* and *B* were exposed for 24 h. The total amount of RNA, as defined by ethidium bromide staining, was increased in stimulated cells.

form appears, and increases over the culture period, peaking at day 3. No 5.4 kb mRNA could be detected even with longer exposures.

Kinetics of the decline in CD45 mRNA encoding high m.w. isoforms following mitogen stimulation. The increase in CD45RA as well as CD45R0 seen at days 1 to 2 of culture is at odds with the molecular analysis. No 5.4 kb mRNA could be detected after 24 h, and yet the increased surface density of CD45RA occurs on nearly all cells. Although this could reflect expression of a determinant on the CD45R0 isoform detected by the CD45RA antibodies used in this study, this seems unlikely as mAb 2H4, which recognizes a determinant on the protein backbone of the CD45RA insert (21), detects the increased density. It is possible that the cells undergoing the transition are stimulated to deplete CD45RA associated with ribosomes by exporting it to the surface for disposal. Alternatively, a general increase in transcription rate of the CD45 gene might yield an initial burst of CD45RA mRNA before the transition in splicing occurs. To address this point, CD45 mRNA was analyzed in T cells stimulated with PHA for times ranging between 2 and 24 h.

In order to overcome the difficulty often encountered in distinguishing the sizes of mRNAs encoding high and low m.w. CD45 isoforms, a probe specific for high m.w. CD45 mRNA was prepared by subcloning an *Sfa* N1 fragment of the pHLC-2 cDNA provided by Dr. Ian Trowbridge (44). This fragment represents most of the sequence encoding exons 4, 5 and 6 of the CD45 gene. The specificity of the probe was confirmed by hybridization on Northern blots of RNA from Raji and HL60 cell lines, which were respectively positive and completely negative for the expression of RNA hybridizing with the *Sfa* N1 fragment (Fig. 3.3 B). Since absolutely no "non-specific"

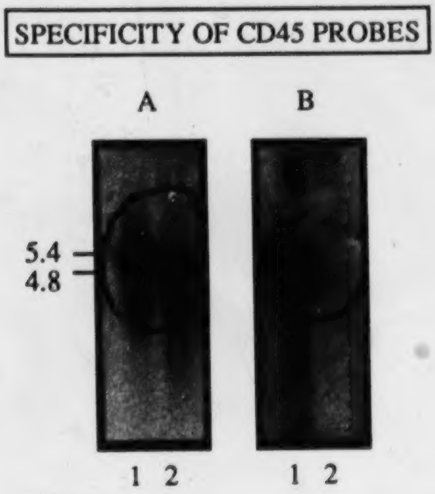


Figure 3.3. Specificity of CD45 probes. In *A*, 2 µg of poly[A⁺] RNA from the Raji (lane 1) and HL60 (lane 2) cell lines was loaded, and the filter probed with the *Bam* H1 fragment of pHLC-1 cDNA (CD45 cytoplasmic region). In *B*, 5 µg of cytoplasmic RNA from Raji (lane 1) and HL60 (lane 2) was loaded, and the filter probed with the *Sfa* N1 fragment of pHLC-2 cDNA (CD45 exons 4-6). CD45 mRNA from the Raji cell line is predominantly high m.w., containing sequences from all 3 variable exons (44,45). CD45 mRNA from the HL60 cell line contains none of the variable region sequences (44).

signals were detected with this probe, the cytodot method was later used with confidence.

PBMC were depleted of CD45R0$^+$ cells and B cells using mAb UCHL1 and B4, respectively, and magnetic beads, as described in Chapter 2. RNA was prepared from 2 x 10^6 unstimulated cells (Fig. 3.4, 0 h) and the remainder were mixed with PHA to 1% final dilution, and aliquoted into wells of 24-well culture plate at 10^6/ml in 2 ml volumes. The contents of one well were removed at each of the time points indicated in Figure 3.4, and RNA was prepared. Accurate quantitation of RNA in each sample was made by fluorometry, and 50 ng was loaded into each well of a cytodot apparatus. The filter was probed with the *Sfa* N1 fragment described above, and the results are shown in Figure 3.4.

There was a transient decrease in the expression of high m.w. CD45 mRNA detected at 2 h following stimulation. By 4 h the level increased significantly beyond that found in resting cells. In this experiment, a high level was sustained until 16 h poststimulation, and then expression decreased by 24 h. The transient initial decrease followed by a rise to above resting-state levels was a feature reproduced in all of 5 separate experiments. The kinetics of the subsequent decline was somewhat variable. In one experiment using nylon-passed spleen cells the drop in expression of high m.w. CD45 mRNA was precipitous after 4 h (Fig. 3.5A). Spleen cells have not been subsequently available to confirm this result. In another experiment using PBMC the decline in high m.w. CD45 mRNA was slower than that depicted in Figure 3.4 (not shown).

KINETICS OF DECLINE IN HIGH M.W. CD45 mRNA AFTER ACTIVATION

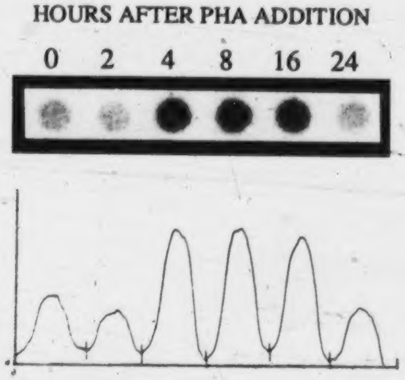


Figure 3.4. Kinetics of the decline in CD45 mRNA encoding high m.w. isoforms following PHA activation. Cytoplasmic RNA was isolated from 2 x 10^6 UCHL1-depleted PBMC activated with 1% PHA for times ranging between 0 and 24 h. *Top* 50 ng RNA was applied to each well of the cytodot apparatus, and the filter was probed with the *Sfa* N1 fragment of pHLC-2 cDNA, detecting only CD45 mRNA that includes exons 4, 5 or 6. The autoradiogram was exposed for 24 h. *Bottom* densitometry scan of the autoradiogram.

As no difficulty was experienced in detecting high m.w. CD45 mRNA in resting PBMC using this probe, it appears that the *Sfa* N1 fragment is more sensitive than the pHLC-1 cytoplasmic-region probe in detecting the high m.w. CD45 mRNAs. At 24 h poststimulation, therefore, mRNA encoding high m.w. CD45 isoforms can sometimes be detected. However, in all experiments the level of expression declined by 24 h, compared with earlier time points.

PHA-stimulated PBL express a variety of mRNAs that segregate roughly into 4 categories based on the kinetics of accumulation (142): 1) mRNAs with detectable expression in unstimulated PBL, with levels increasing or remaining the same following stimulation; 2) mRNAs with detectable expression in unstimulated PBL, with levels that initially decline and then rise; 3) mRNAs with undetectable expression in unstimulated PBL, and a rapid increase post-stimulation; and 4) mRNAs with undetectable expression in unstimulated PBL, and delayed accumulation several hours after stimulation. mRNA encoding high m.w. forms of CD45 clearly fall into the second category. In view of the work linking CD45 to TCR-mediated activation (26,55,99), and to activation of pp56^{lck} (62), it is interesting to note that genes encoding the TCR β-chain and pp56^{lck} are among the genes reported to have similar post-stimulation kinetics (143,144). In addition, the *pim-1* oncogene is a putative tyrosine-kinase with a similar expression pattern in activated T cells (142). Recently, Paillard et al (144) demonstrated that under conditions of maximal activation, mRNAs encoding CD4, CD8 and TCR-α, as well as TCR-β were all transiently down-regulated. With the exception of the *pim-1* product, all of the above molecules have been reported to be associated in some way with the CD3/TCR complex. The significance of these observations is not understood at present.

CD45 mRNA encoding high m.w. isoforms is superinduced by cycloheximide. The decline in high m.w. CD45 mRNA during the first 24 h of stimulation suggests that an active degradation process may be involved. To test this, T cells were stimulated in the presence or absence of cycloheximide, an inhibitor of protein synthesis. If CD45 mRNA is degraded by an RNase, interruption of the enzyme's production should allow unimpeded accumulation of the RNA.

Spleen cells were passed over a nylon wool column to deplete B cells and RNA was prepared from 2 x 10^7 unstimulated cells (Fig. 3.5, 0 h). The remainder were mixed with PHA to 1% final dilution, and aliquoted into small culture flasks at 10^6/ml in 20 ml volumes. Cycloheximide at 20 µg/ml was added to a duplicate set of cultures.The contents of one flask from each set were removed at each of the time points indicated in Figure 3.5, and RNA was prepared. Accurate quantitation of RNA in each sample was made by fluorometry, and 1 µg was loaded into each well of the cytodot apparatus. The filter was probed with the CD45 *Sfa* N1 fragment , and the results are shown in Figure 3.5. In Figure 3.5A, the kinetics of the transition of CD45 mRNA encoding high m.w. isoforms is shown. The initial transitory decrease in expression followed by a rise above resting-state levels is again evident. However, after 4 h the level of message drops dramatically. In contrast, in the presence of cycloheximide high m.w. CD45 mRNA continues to accumulate up to 24 hours (Fig. 3.5B). Superinduction of high m.w. CD45 mRNA by cycloheximide confirms the involvement of an active degradation process in the poststimulation decline of the mRNA. Such mechanisms are known to be involved in the degradation of other inducible mRNAs; for example, that coding for IL-2.

SUPERINDUCTION OF HIGH M.W. CD45 mRNA BY CYCLOHEXIMIDE

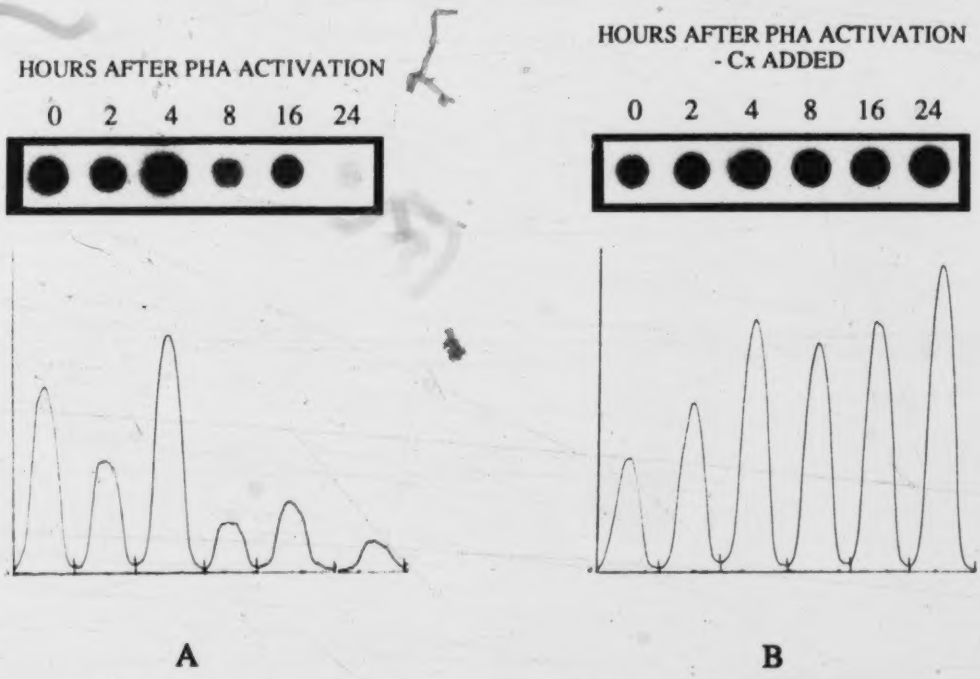


Figure 3.5. CD45 mRNA coding for high m.w. isoforms is superinduced by cycloheximide. In *A*, cytoplasmic RNA was isolated from 2 x 10^7 B cell-depleted spleen cells activated with 1% PHA for times ranging between 0 and 24 h. In *B*, 20 μg/ml cycloheximide was added to a duplicate set of cultures at - 0.5 h. *Top* 1 μg RNA was applied to each well of the cytodot apparatus, and the filter was probed with the *Sfa* N1 fragment of pHLC-2 cDNA, detecting only CD45 mRNA that includes exons 4, 5 or 6. The autoradiogram was exposed for 48 h. *Bottom* densitometry scans of the autoradiogram.

Decline of CD45 mRNA encoding high m.w. isoforms is delayed in stimulated CD3$^-$4$^-$8$^-$ thymocytes. One prediction of the hypothesis, detailed in chapter 1, that expression of CD45RA marks the generative thymic lineage and is essential for the survival of thymocytes, is that the shift from high to low m.w. CD45 would not occur in thymocytes following stimulation, unless such stimulation initiated a programmed cell death. The signals and conditions required for cell survival in the thymus, particularly among cells which have not yet expressed TCR, are still undefined. I have repeatedly observed that CD3$^-$4$^-$8$^-$ thymocytes have significantly reduced viability following 24 h culture in 1% PHA. This suggests that these immature cells may be sensitive to overstimulation and that 1% PHA may initiate programmed cell death in a manner analogous to that presumed to occur *in vivo.* Therefore, an alternative method of *in vitro* stimulation was used in order to analyse the kinetics of CD45 mRNA transitions in CD3$^-$4$^-$8$^-$ thymocytes. It was learned during an extensive analysis of the proliferative potential of CD3$^-$4$^-$8$^-$ thymocytes (vide infra, Chapter 5) that a combination of mitogenic anti-CD2 mAb, together with anti-CD28 and 1 ng/ml PMA, was required for maximal proliferation of these cells. Therefore the same conditions were used to stimulate CD3$^-$4$^-$8$^-$ thymocytes for analysis of CD45 mRNA.

RNA was prepared from 2 x 10^6 unstimulated CD3$^-$4$^-$8$^-$ thymocytes (Fig. 3.6, 0 h) and the remainder were mixed with mAb 9.1, 9.6 and 9.3, each at 1 µg/ml, and 1 ng/ml PMA, and aliquoted into wells of a 24-well culture plate at 10^6/ml in 2 ml volumes. The contents of one well were removed at each of the time points indicated in Figure 3.6, and RNA was prepared. Accurate quantitation of RNA in each sample was made by fluorometry, and 200 ng was loaded into each well of the cytodot apparatus. The filter was probed with the *Sfa* N1 fragment described above, and the results are shown in Figure 3.6.

KINETICS OF DECLINE IN HIGH M.W. CD45 mRNA
AFTER ACTIVATION OF CD3-4-8- THYMOCYTES

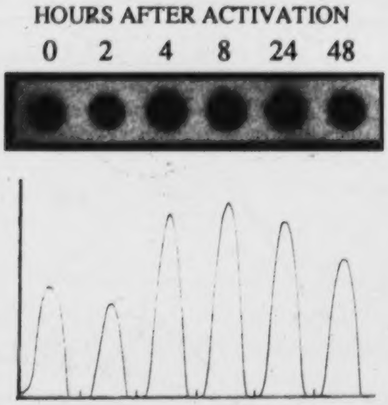
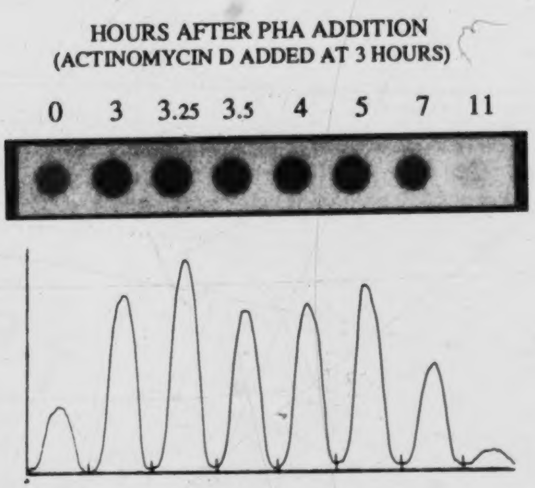


Figure 3.6. Decline of CD45 mRNA encoding high m.w. isoforms is delayed in activated CD3$^-$4$^-$8$^-$ thymocytes. Cytoplasmic RNA was isolated from 2 x 10^6 CD3$^-$4$^-$8$^-$ thymocytes cultured with mAb 9.1 + 9.6 + 9.3 (anti-CD2 + -CD28), each at 1 µg/ml, and 1 ng/ml PMA, for times ranging between 0 and 48 h. *Top* 200 ng RNA was applied to each well of the cytodot apparatus, and the filter was probed with the *Sfa* N1 fragment of pHLC-2 cDNA, detecting only CD45 mRNA that includes exons 4, 5 or 6. The autoradiogram was exposed for 24 h. *Bottom* densitometry scan of the autoradiogram.

There was a transitory initial decrease in expression of high m.w. CD45 mRNA, as also seen in activated PBMC. However, although the level of message began to decline at 24 h, it was still well above resting-state levels even at 48 h poststimulation. Thus, there appears to be a significant difference between mature T cells and immature thymocytes in the kinetics of the shift from high to low m.w. CD45 mRNA, consistent with the hypothesis that CD45RA is an essential element in the survival of thymocytes.

Degradation of CD45 mRNA encoding high m.w. isoforms. The superinduction by cycloheximide of high m.w. CD45 mRNA suggests that a labile protein is involved in its degradation. Stabilization of mRNA by cycloheximide is characteristic of many cytokine genes with a short messenger half-life, including IL-2, TNF, and GM-CSF. In order to estimate the half-life of high m.w. CD45 mRNA, PBMC were first enriched for CD45RA$^+$ cells by depletion with mAb UCHL1, and then stimulated with 1% PHA for 3 hours to enhance the expression of high m.w. CD45 mRNA before adding actinomycin D to stop transcription. Samples were taken at 15 min, 30 min, 1 h, 2 h, 4 h and 8 h thereafter, for RNA preparation. Exactly 30 ng of RNA from each sample was dotted onto a nylon membrane and the filter was probed with the *Sfa* N1 fragment of pHLC-2. The results are shown in Figure 3.7. There appeared to be a lag phase of about 2 h before the onset of decay of high m.w. CD45 mRNA, suggesting that the enzyme(s) involved in the degradation pathway is (are) induced following stimulation. The $t_{1/2}$ was estimated at 2.25 h by linear regression analysis of the data from 2 h and later (Fig. 3.9). Estimations of $t_{1/2}$ using actinomycin D are potentially flawed by the non-specificity of its inhibitory effect on transcription. Inhibition of the production of a labile protein involved in degrading mRNA may lead to an over-estimation of its $t_{1/2}$. Thus, the $t_{1/2}$ of CD45 mRNA may be shorter than estimated.



DEGRADATION OF HIGH M.W. CD45 mRNA

Figure 3.7. Degradation of CD45 mRNA encoding high m.w. isoforms. UCHL1-depleted PBMC were cultured at 10^6/ml with 1% PHA for 3 h, then 5 µg/ml actinomycin D was added and samples taken at times ranging between 15 min and 8 h. *Top* 30 ng RNA was applied to each well of the cytodot apparatus, and the filter was probed with the *Sfa* N1 fragment of pHLC-2 cDNA, detecting only CD45 mRNA that includes exons 4, 5 or 6. The autoradiogram was exposed for 24 h. *Bottom* densitometry scan of the autoradiogram.

Degradation of CD45R0 mRNA. CD45RA is lost from the surface of antigen-inexperienced T cells following antigenic or mitogenic stimulation, whereas CD45R0 is permanently expressed once induced. Expression of high m.w. CD45 mRNA rapidly declines after activation, whereas CD45R0 message accumulates to, and remains at, a relatively high level following the shift to its expression (Fig. 3.2). These observations raised the question of a possible difference in the half-life of the high and low m.w. CD45 mRNAs, despite the fact that such a difference would be unlikely since all species of CD45 mRNA presumably share an identical 3' untranslated region. Consensus AU-rich sequences in this region of other inducible genes have been implicated in the degradative pathway of unstable mRNAs (145-147), and several AU-rich sequences can be found also in the 3' untranslated region of CD45 mRNA.

To determine the degradation rate of CD45R0 mRNA, PBMC were first stimulated for 3 days with 1% PHA, to ensure that high m.w. CD45 mRNA would be undetectable, before adding actinomycin D. This was necessary because it is not possible to probe specifically for CD45R0 mRNA since it has no unique sequence. Samples were taken for RNA preparation at intervals up to 24 h after actinomycin D addition, and 200 ng from each sample was dotted onto a nylon filter and probed with pHLC-1 cDNA, which hybridizes with part of the sequence encoding the cytoplasmic region of CD45 (44) (Fig.3.8). The filter was later stripped and reprobed with the *Sfa* N1 fragment of pHLC-2 cDNA to confirm the absence of high m.w. CD45 mRNA (not shown). The $t_{1/2}$ of CD45R0 mRNA was estimated at 3.5 h by linear regression of data from all time points (Fig.3.9). It remains to be determined whether or not the estimated half-lives of high and low m.w. CD45 mRNA are significantly different.

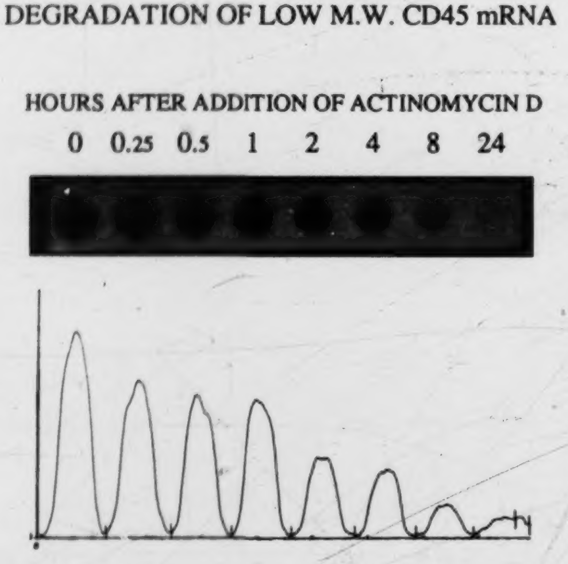


Figure 3.8. Degradation of CD45 mRNA encoding CD45R0. 2 x 10^7 PBMC were cultured for 3 days with 1% PHA and then 5 µg/ml actinomycin D was added. Samples were taken at intervals up to 24 h thereafter. *Top* 200 ng RNA was applied to each well of the cytodot apparatus, and the filter was probed with pHLC-2 cDNA. The autoradiogram was exposed for 24 h. *Bottom* densitometry scan of the autoradiogram.

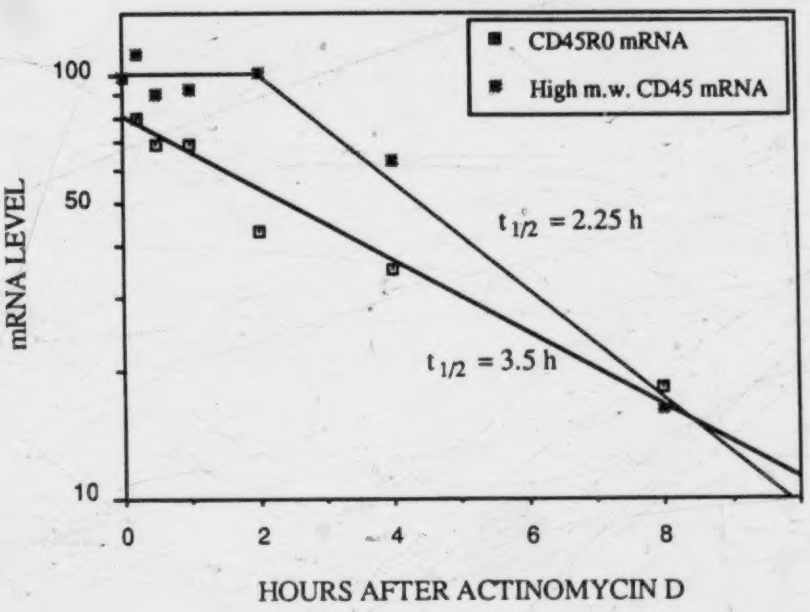


Figure 3.9. $t_{1/2}$ of CD45 mRNA. Data from experiments shown in figures 3.7 and 3.8 were used to estimate the $t_{1/2}$ of both high and low m.w. CD45 mRNA. The data from both experiments are normalized to 100 at the time of actinomycin D addition. The $t_{1/2}$ of low m.w. CD45 mRNA was obtained by linear regression of all data. The value shown for high m.w. CD45 mRNA was obtained using only data at 2 hours and later, because there appeared to be a lag phase before the apparent decay.

SUMMARY

The kinetics of the transition from high to low m.w. CD45 mRNA has been investigated. We have found that there is an immediate decrease in the level of high m.w. CD45 mRNA following mitogen stimulation of T cells, quickly followed by an increase above steady-state levels. This is consistent with the transitory increase in cell-surface density of CD45RA observed at 1-2 days poststimulation. Within 24 h of stimulation the level of high m.w. CD45 mRNA begins to decline permanently, and low m.w. CD45 mRNA is expressed. In mitogen-stimulated cells the $t_{1/2}$ of high and low m.w. CD45 mRNA was estimated at 2.25 h and 3.5 h respectively. It remains to be determined whether or not these values are significantly different. The relatively short half-life of high m.w. CD45 mRNA is controlled by a labile degradation pathway that itself may be induced, as suggested by a lag phase prior to the onset of decay of high m.w. CD45 mRNA in stimulated CD45RA$^+$ T cells. A system that is responsive to the cellular microenvironment is likely to be labile; however it is perhaps surprising that CD45R0 mRNA is also relatively short-lived, since it appears to be permanently expressed once induced. It will be interesting to determine the $t_{1/2}$ of both high and low m.w. CD45 mRNA in resting T cells, in view of the possibility that the degradation process is induced only after cellular activation.

Finally, we have found a prolonged presence of high m.w. CD45 mRNA in CD3$^-$4$^-$8$^-$ thymocytes following stimulation by CD2 and CD28 mAb. It remains to be determined whether this is an intrinsic property of immature T cells, or a reflection of the method used to stimulate them. The point may be moot if *in vitro* activation via CD2 and CD28 relates to physiologic stimuli *in vivo*. Interaction between CD2 on thymocytes and its ligand, LFA-3, on thymic epithelial cells, has been demonstrated (149), however, the CD28 ligand has yet to be identified.

IV. CD45RA AS A PRIMARY SIGNAL TRANSDUCER STIMULATING IL-2 AND IL-2R mRNA SYNTHESIS BY HUMAN PROGENITOR THYMOCYTES

It is generally agreed that the least mature cells in the human thymus are $CD3^-4^-8^-$ "triple negative" "pro-T" cells (150); however, the factors driving proliferation and differentiation of these cells are largely unknown. Of the surface molecules known to be present on pro-T cells, CD2 is the best characterized; it is known to transmit activation signals to mature T cells (118) and thymocytes (151,152), and interaction between the CD2 molecule on thymocytes and its ligand, LFA-3, on thymic epithelial cells has been demonstrated (149). However, there is as yet no consensus on the role of CD2 in the growth and maturation of early thymocytes. Some investigators have provided evidence that CD2 is expressed after the IL-2R during murine thymocyte ontogeny (153,154), and in the human system, cytoplasmic CD3 appears to be expressed before CD2 (150). These latter observations suggest that CD2 may not be involved in the early development of T cells. Thus, a search for alternative signaling molecules present on progenitor thymocytes is an important area of investigation.

As discussed in Chapter 1, antibodies reacting with either restricted or common epitopes of the CD45 molecule will inhibit or enhance, depending on the conditions used, the proliferative response of mature peripheral blood T cells induced by a variety of stimuli (26,94-97,99). To study the signaling potential of CD45 molecules on progenitor thymocytes, we chose to use the detection of IL-2 and IL-2R mRNA as relatively early indicators of cellular activation, rather than proliferation assays in which the late read-out may reflect activation of cells that have differentiated during the culture period. A reasoned assumption was made that cells within the generative intrathymic lineage will have greater proliferative potential than those which have been committed to intrathymic death, and that the IL-2/IL-2R interaction is involved in proliferation of thymocytes (3,155-158) as well as in the proliferation of mature T cells.

We first compared the ability of unfractionated thymocytes, CD45R0-depleted thymocytes, and the CD3$^-$4$^-$8$^-$ subset, to respond to mitogenic stimulation *in vitro* by Northern analysis of IL-2 and, in some experiments, IL-2R mRNA. Evidence is then provided that CD45RA is involved in transmembrane signalling in CD3$^-$4$^-$8$^-$ thymocytes, by the demonstration of synergy between suboptimal doses of mitogen and antibodies against CD45RA in the induction of IL-2 mRNA.

RESULTS

CD45 phenotype analysis of thymus populations. In order to assess the role of CD45RA in the triggering of thymocytes, we required populations of thymocytes enriched for cells expressing CD45RA. As previously described, CD45R0-depleted thymocyte fractions include predominantly CD45RA$^+$ cells (9). This is confirmed in the experiments shown in Table 4.1 which also shows that CD3$^-$4$^-$8$^-$ thymocytes are enriched for cells expressing CD45RA. CD3$^-$4$^-$8$^-$ CD45R0$^-$ fractions have a predominantly CD45RA$^+$ phenotype and have a higher density of CD45RA, compared to total CD3$^-$4$^-$8$^-$, as defined by brightness of IF (Table 4.1). CD45R0$^-$ thymocytes include all of the major subsets of thymocytes defined by CD3, CD4 and CD8 (Table 4.2). This fraction is enriched for CD4 and CD8 single positives and for the multinegative CD3$^-$4$^-$8$^-$ subset.

Phenotype analysis of CD3$^-$4$^-$8$^-$ thymocytes. CD3$^-$4$^-$8$^-$ CD19$^-$ thymocytes, prepared by depletion with mAb as described (Chapter 2), are 93% CD45$^+$, defining them as bone

TABLE 4.1

CD45 phenotypes in thymic subsets

	% Positive cells		
	CD45	**CD45RA**	**CD45R0**
Unfractionated	89±11	20±8	83±9
CD45R0$^-$	87±4	70±22	3±3
CD3$^-$4$^-$8$^-$	93±4	78±14	9±4
CD3$^-$4$^-$8$^-$[a]	92	84	11
CD3$^-$4$^-$8$^-$ CD45R0$^-$[a]	85	77	2

Phenotype was determined by single indirect IF on depleted populations using mAbs ASH1621, BT25 and UCHL1. Depletions were done using OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), and/or UCHL1, always including FMC63 (CD19) to deplete thymic B cells.

[a] Cell populations used for the experiment described in Figure 4.4, both derived from the same thymus.

TABLE 4.2

CD3, CD4 and CD8 phenotypes of CD45R0$^-$ thymocytes

% Positive thymocytes			
CD4$^-$8$^-$	35	CD3$^-$, 4$^-$8$^-$	32
CD4$^-$8$^+$	9	CD3$^+$, 4$^-$8$^-$	1
CD4$^+$8$^-$	27	CD3$^-$, 4$^+$ and/or 8$^+$	7
CD4$^+$8$^+$	29	CD3$^+$, 4$^+$ and/or 8$^+$	59

Subset distribution of the CD45R0$^-$ fraction used in Figure 4.1 was determined using double IF with CD4-PE and CD8-FITC, or with CD3-FITC and a mixture of CD4-PE and CD8-PE. Electronic gates for analysis were set based on staining of an aliquot of cells with isotype-matched control antibodies.

marrow-derived cells, and include fewer than 4% CD3+, CD4+, CD8+ or B cells (Fig. 4.1). They are predominantly CD2$^+$ and CD45RA$^+$, and 41% CD1+ (Fig.4.1).

IL-2 and IL-2R mRNA induction in mitogen stimulated thymocytes. The initial experiments were designed to assess the ability of human thymocytes to induce the expression of IL-2 and IL-2R mRNA under optimal conditions (Fig. 4.2). Each of the three thymus cell populations (unfractionated, CD45R0$^-$ and CD3$^-$4$^-$8$^-$) were cultured for 24 h with either a combination of PHA and PMA, or PMA and Ionomycin. Ten percent Con A sup was also added to all cultures. Total cytoplasmic RNA from 2 x 10^6 cells was extracted and run on Northern gels. Ethidium bromide staining of the gels indicated that each lane contained similar amounts of RNA. The results (Fig. 4.2) show that 1) IL-2 and IL-2R mRNA were not detected in unstimulated thymocytes (lanes1, 4 and 7); 2) At the concentrations used, the combination of PHA and PMA (lanes 2, 5 and 8) was much more efficient at activating all 3 cell populations than PMA and Ionomycin (lanes 3,6 and 9); and 3) Both CD45R0$^-$ and CD3$^-$4$^-$8$^-$ fractions were greatly enriched over unfractionated thymocytes for cells which could be induced to make IL-2 mRNA. Densitometry of the autoradiogram indicates differences of at least 10-fold. The differences in IL-2R mRNA levels are qualitatively similar, although less dramatic. The amount of IL-2 mRNA from stimulated CD3$^-$4$^-$8$^-$ thymocytes was greater than that from CD45R0$^-$ thymocytes suggesting than most of the activity in the latter subset derived from the enriched proportion of multi-negatives (20-60%, Table 4.2; 9,10).

MAbs to CD45 determinants stimulate IL-2 and IL-2 mRNA induction in CD3$^-$4$^-$8$^-$ thymocytes. Since early thymocytes lack CD3/TCR complex but are still responsive to stimulation by PHA which is thought to act through the TCR, we speculated that an

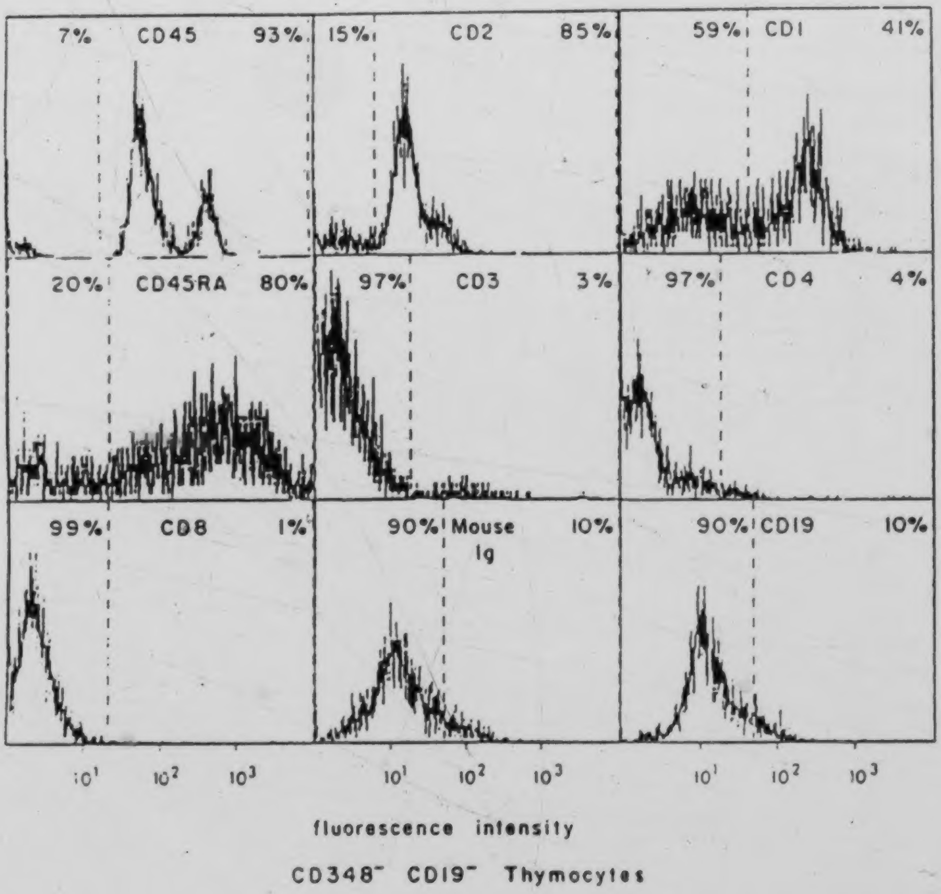


Figure 4.1. Phenotype of $CD3^-4^-8^-$ thymocytes. CD45, CD2, CD1, CD45RA, CD3, CD4, and CD8 were detected by direct IF using FITC- or PE-conjugated HLE 1, Leu 5, T6, 3AC5, Leu 4, Leu 3 and Leu 2, respectively. CD19 was detected by indirect IF using FMC 63 and FITC-conjugated anti-mouse Ig. The vertical axis represents number of cells, and the horizontal axis log fluorescence intensity. Results were read on the FACSCAN.

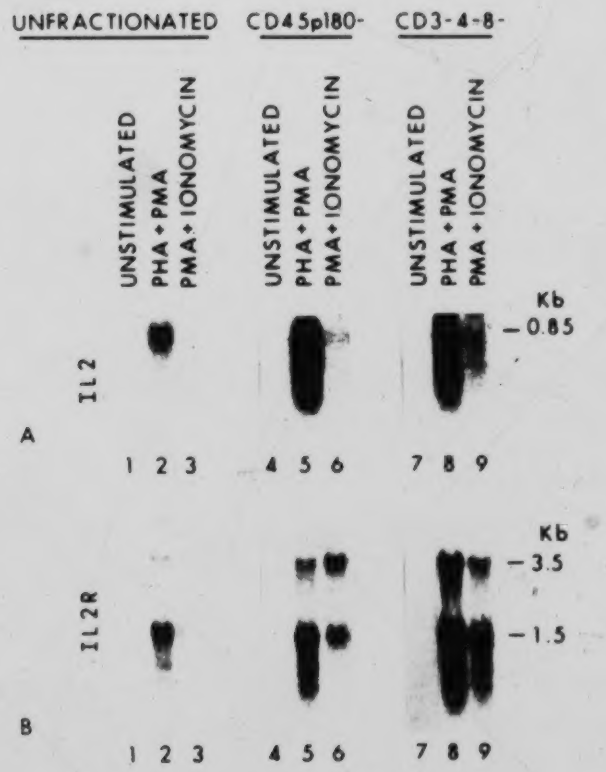

UNFRACTIONATED
CD45pl80-
CD3-4-8-
UNSTIMULATED
PHA + PMA
PMA + IONOMYCIN
UNSTIMULATED
PHA + PMA
PMA + IONOMYCIN
UNSTIMULATED
PHA + PMA
PMA + IONOMYCIN
Kb
— 0.85
IL2
A
1 2 3
4 5 6
7 8 9
Kb
— 3.5
— 1.5
IL2R
B
1 2 3
4 5 6
7 8 9

Figure 4.2. Enhanced stimulation of IL-2 and IL-2R mRNA in CD45R0-depleted and $CD3^-4^-8^-$ thymocyte fractions. Northern blots of total cytoplasmic RNA from unfractionated, $CD45R0^-$ and $CD3^-4^-8^-$ thymus fractions, either unstimulated (lanes 1, 4, 7) or stimulated for 24 h with 1% PHA and 20 ng/ml PMA (lanes2, 5, 9), or 1ng/ml PMA and 125 ng/ml Ionomycin (lanes 3, 6, 9) were probed with A) IL-2 cDNA and B) IL-2R cDNA. RNA derived from approximately 2 x 10^6 cells was loaded in each lane. In 4 of 5 experiments, no IL-2 mRNA was detectable from unfractionated thymocytes after stimulation with either PHA/PMA or PMA/Ionomycin. In all experiments, the amount of IL-2 mRNA was greatly enriched in $CD3^-4^-8^-$ thymocytes and, to a lesser extent, in $CD45R0^-$ thymocytes.

alternate cell surface molecule must be involved in signal transduction. The experiment of Figure 4.3 demonstrates that mAb to CD45 epitopes efficiently synergized with suboptimal PHA and PMA to induce the synthesis of IL-2 and IL-2R mRNA. The levels of IL-2 mRNA induced in $CD3^-4^-8^-$ thymocytes by 1% PHA either with or without TCGF and PMA are compared in Figure 4.3A, lanes 3, 4 and 5. Lanes 6 and 7 show the result of supplementing a suboptimal dose of PHA and PMA with a mixture of 4 monoclonal antibodies directed against CD45; three of the antibodies used recognize epitopes specifically on CD45RA (FMC44, BT25 and 3AC5), while one was directed against a CD45 common determinant (ASH1621). The addition of the antibody mixture clearly synergized with the suboptimal dose of PHA and PMA to enhance IL-2 mRNA expression. There also appears to be a marginal increase in IL-2R mRNA expression upon addition of CD45 antibodies (compare lanes 6 and 7, Fig. 4.3B). It can also be seen that cells stimulated with the higher PHA/PMA dose contained less IL-2R mRNA than those stimulated with the lower dose (lanes 5 and 6, Fig. 4.3B). I have consistently found that $CD3^-4^-8^-$ thymocytes, unlike PBL or unfractionated thymocytes, have reduced 24 h viability in the presence of 1% PHA, compared to cells cultured in lower doses of PHA or without mitogen. Therefore, it is likely that by 24 h in culture these cells have passed the peak of IL-2R mRNA production.

MAb to CD45RA but not to CD45 common determinants stimulate $CD3^-4^-8^-$ thymocytes. To determine which epitopes of the CD45 molecule were responsible for the synergistic stimulation of mRNA synthesis, the antibodies were tested individually. CD45RA-specific mAbs FMC44, BT25 and 3AC5 all enhanced IL-2 mRNA expression (Fig. 4.4, lanes 3, 4 and 6) while 1621, which recognized a determinant common to all CD45 isoforms, did not (lane 5). The lack of a response to mAb 1621 also provides an internal negative control demonstrating the specificity of the

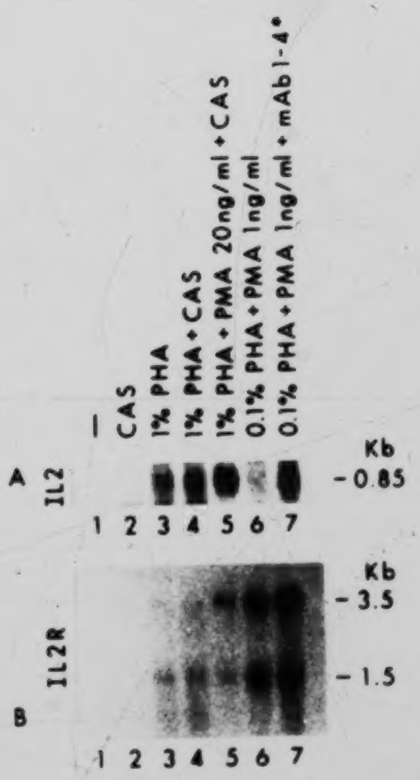

—
CAS
1% PHA
1% PHA + CAS
1% PHA + PMA 20ng/ml + CAS
0.1% PHA + PMA 1ng/ml
0.1% PHA + PMA 1ng/ml + mAb1-4*
A
IL2
Kb
-0.85
1 2 3 4 5 6 7
Kb
-3.5
-1.5
IL2R
B
1 2 3 4 5 6 7

Figure 4.3. Synergistic triggering of IL-2 and IL-2R mRNA by anti-CD45 mAbs and suboptimal PHA and PMA in CD3$^-$4$^-$8$^-$ thymocytes. Northern blots of total cytoplasmic RNA from CD3$^-$4$^-$8$^-$ thymocytes, unstimulated (lane 1) or cultured for 24 h with various agents as indicated (lanes 2-7) were probed with A) IL-2 cDNA or B) IL-2R cDNA. RNA from 2 x 10^6 cells was loaded in each lane and ethidium bromide staining indicated equivalent amounts of RNA. CAS (ConA supernatant) was prepared from PBL stimulated with ConA for 3 days.

*mAb 1-4 = mixture of mAbs FMC44, BT25, ASH1621 (1/1000 dilution of ascites fluid) and 3AC5 at 1μg/ml.

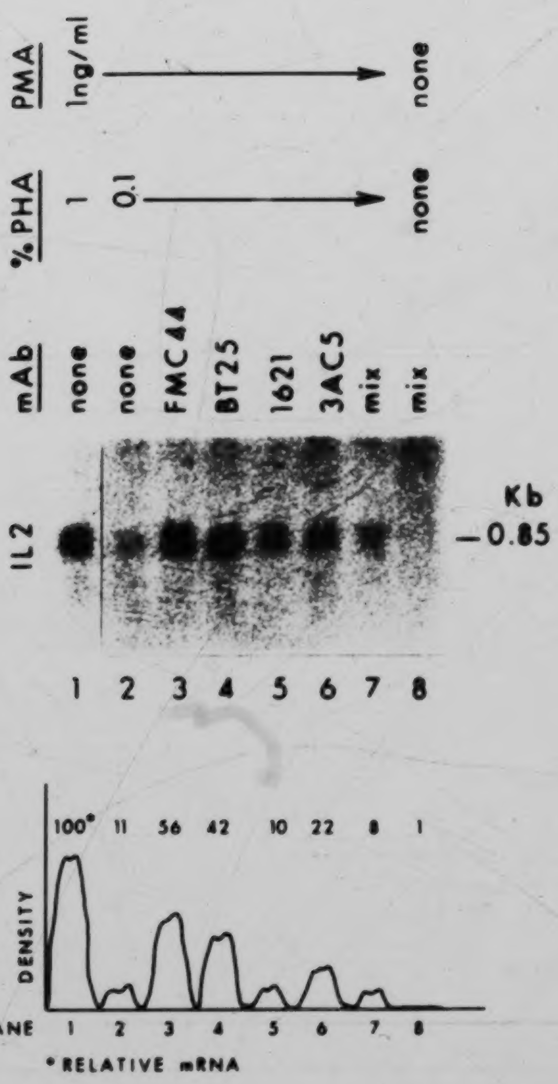


Figure 4.4. MAb to CD45RA but not to CD45 common determinants stimulate the synthesis of IL-2 mRNA in $CD3^-4^-8^-$ thymocytes. *Top* Northern blot of total cytoplasmic RNA from $CD3^-4^-8^-$ thymocytes cultured for 24 h under conditions indicated; probed with IL-2 cDNA. RNA from 2 x 10^6 cells was loaded in each lane. Ethidium bromide staining indicated equivalent amounts of RNA in each lane. *Bottom* Densitometry scan of autoradiogram shown above. mix = mixture of CD45RA-specific and CD45-specific antibodies as described for Figure 4.2.

stimulation by mAbs to CD45RA. Unexpectedly, addition of all four antibodies together resulted in no detectable IL-2 mRNA expression (lane 7) in contrast to the result shown in Figure 4.3. However the experiment shown in Figure 4.3 did not include antibodies added individually making these difficult to compare. In Figure 4.4, it appears that the presence of mAb 1621 was inhibitory to the signals transduced through CD45RA.

The response to anti-CD45RA is disproportionately enhanced in CD3^{-}4^{-}8^{-} CD45R0^{-} thymocytes. CD3^{-}4^{-}8^{-} thymocytes include from 7-12% CD45R0^{+} cells (10). To further assess the effects of CD45RA mAb, we stimulated thymocytes depleted of cells bearing CD3, CD4, CD8 and CD45R0 (Fig. 4.5). Whereas CD3^{-}4^{-}8^{-} cells have peaks of CD45RA IF at channels 75 and 105, the CD3^{-}4^{-}8^{-} CD45R0^{-} population includes more cells with a high density of CD45RA (IF at channel 165). In this experiment we included a test for the effect of soluble anti-CD45RA alone, and found that it cannot induce the expression of IL-2 mRNA in CD3^{-}4^{-}8^{-} thymocytes (Fig. 4.5, lane 7). In addition, the response to anti-CD45RA in the presence of suboptimal PHA/PMA was shown to be dose dependent: a 1/1000 dilution of FMC44 produces an IL-2 mRNA approximately 75% of that induced by the same antibody at 1/500 dilution (lanes 8 and 9, compared by scanning densitometry of the autoradiogram). The CD3^{-}4^{-}8^{-} thymocyte population used in this experiment included about 11% CD45R0^{+} cells. When these cells were removed by depletion with mAb UCHL1 it was found that the remaining cells were significantly more responsive to activation with anti-CD45RA mAb than were CD3^{-}4^{-}8^{-} thymocytes (Fig. 4.5, lanes 14 and 15, versus lanes 8 and 9). The CD45 phenotypes of the 2 subsets are shown in Table 4.1. Depletion of the CD45R0^{+} cells from the CD3^{-}4^{-}8^{-} population apparently enriched for stromal cells, since CD45^{+} cells dropped from 92% to 85%. The proportion of CD45^{+} cells which were CD45RA^{+}

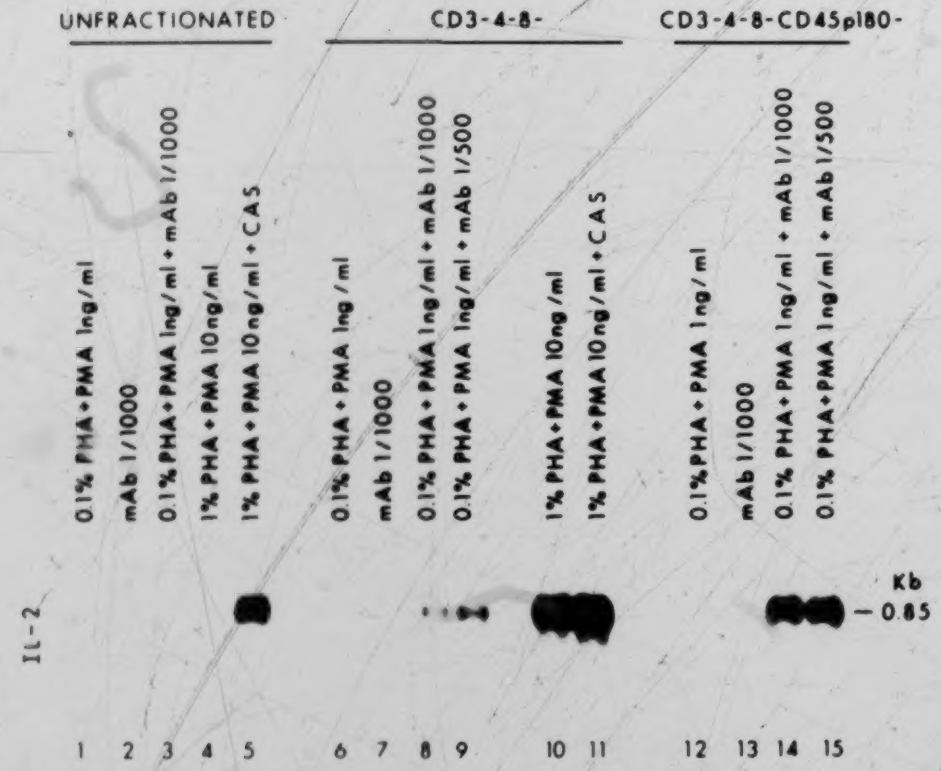

UNFRACTIONATED
CD3-4-8-
CD3-4-8-CD45pl80-
0.1% PHA+PMA 1ng/ml
mAb 1/1000
0.1% PHA+PMA 1ng/ml + mAb 1/1000
1% PHA+PMA 10ng/ml
1% PHA+PMA 10ng/ml + CAS
0.1% PHA + PMA 1ng/ml
mAb 1/1000
0.1% PHA+PMA 1ng/ml + mAb 1/1000
0.1% PHA + PMA 1ng/ml + mAb 1/500
1% PHA+PMA 10ng/ml
1% PHA+PMA 10ng/ml + CAS
0.1% PHA + PMA 1ng/ml
mAb 1/1000
0.1% PHA+PMA 1ng/ml + mAb 1/1000
0.1% PHA+PMA 1ng/ml + mAb 1/500
IL-2
Kb
— 0.85
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.5. Enhanced synthesis of IL-2 mRNA after triggering of CD3$^-$4$^-$8$^-$ CD45R0$^-$ thymocytes by anti-CD45RA mAb. Northern blots of total cytoplasmic RNA from unfractionated, CD3$^-$4$^-$8$^-$ and CD3$^-$4$^-$8$^-$ CD45R0$^-$ thymocytes, cultured for 24 h under conditions indicated; probed with IL-2 cDNA. RNA from 2 x 10^6 cells was loaded in each lane. The amount of IL-2 mRNA in lanes 14 and 15 was approximately 4-fold greater than that in lanes 8 and 9 as measured by densitometry of the autoradiograph. Equivalent amounts of RNA were loaded in each lane as determined by ethidium bromide staining. CAS was as described in the legend to Figure 4.3.

was similar before and after depletion of CD45R0$^+$ cells; however, after depletion the remaining CD45RA$^+$ cells expressed a higher density of CD45RA as defined by brighter IF, suggesting that depletion of CD45R0$^+$ cells removed transitional thymocytes (9) coexpressing low levels of CD45RA as well as CD45R0.

DISCUSSION

We have proposed that the expression of high m.w. isoforms of CD45 is essential for growth and differentiation of immature T cells, and that inappropriate rearrangement or specificity of TCR activates a transition from expression of CD45RA to that of CD45R0 resulting in intrathymic death (9,10). The experiments presented in this chapter were designed to test one prediction of our hypothesis that the intrathymic generative lineage is defined by selective expression of high m.w. isoforms of CD45. The ability of thymocytes to express IL-2 and IL-2R mRNA was used as an indicator of functional potential, and correlated with the expression of CD45RA. We found that when total thymocytes were enriched for CD45RA$^+$ cells by depletion of CD45R0$^+$ cells, the resulting population had an enhanced ability to express IL-2 and IL-2R mRNA. Since depletion of CD45R0$^+$ cells removes nearly all of the CD4$^+$8$^+$ cortical cells and enriches for CD4$^+$8$^-$ and CD4$^-$8$^+$ medullary cells, as well as CD3$^-$4$^-$8$^-$ immature cells, this result is to be expected based on the assumption that IL-2 plays an important role in thymic development.

We have shown that CD3$^-$4$^-$8$^-$ human thymocytes can be stimulated with PHA plus PMA to express IL-2 and IL-2R genes. In contrast, CD4$^-$8$^-$ thymocytes from adult mice fail to produce IL-2 after stimulation with Con A plus PMA, although they do synthesize IL-2 when the Ca ionophore A23187 is substituted for Con A (159). This is generally believed to be due to the fact that the mitogen Con A acts by binding to, and triggering through, the TCR, which is absent on the majority of murine double negative cells (159,160). The mitogen PHA also stimulates T cells by binding to cell surface proteins, particularly the TCR. However, since the TCR is absent from these early thymocytes, PHA stimulation must occur through alternative structures. CD2 and CD45RA are candidates for such structures, since both have been shown to have signaling properties in mature T cells (26,118), and anti-CD45RA was able to synergize with suboptimal PHA in the stimulation of mature T cells (26). In peripheral blood T cells, signaling via CD2 is tightly linked to the CD3 activation pathway, since modulation of CD3 from the cell surface prevents anti-CD2-induced activation. One might expect, therefore, that CD2-induced activation would be defective in $CD3^-$ thymocytes. There are several reports that even $CD3^+$ thymocytes and cord T cells are unable to proliferate to anti-CD2 stimuli in the absence of accessory signals, in contrast to mature peripheral blood T cells (151,161). Although Yang et al. reported that $CD4^-8^-$ thymocytes did not proliferate to mitogenic CD2 mAb in combination with anti-CD28 (162), Denning et al. showed that $CD3^-4^-8^-$ thymocytes proliferated well to mitogenic anti-CD2 in the presence of PMA or IL-2 (158). Toribio et al. have demonstrated moderate proliferation of $CD3^-4^-6^-8^-$ thymocytes in response to either anti-CD2 or anti-CD28, even in the absence of any co-stimulator (157). Together these observations suggest that CD2 molecules on $CD3^-$ thymocytes are functional, but that they may operate differently from those on mature T cells. Interestingly, Kyewski et al. found that although the presence of anti-CD2 in fetal thymus organ cultures significantly decreased the cell-surface expression of CD2, the development of all normal subsets of thymocytes was unaffected (163). Therefore, the role of CD2 in early thymocyte development remains uncertain.

Our experiments clearly demonstrate that anti-CD45RA mAb can restore the ability of suboptimally stimulated CD3$^-$4$^-$8$^-$ thymocytes to synthesize IL-2 and IL-2R mRNA. Thus, it appears that CD45RA molecules can indeed transmit environmental signals to the cell interior. We propose that CD45RA represents a primary signal transducer for thymocytes. It has been recently reported that in mature T cells CD45 molecules modify signals transmitted via other cell surface molecules, including CD2 (23,99), raising the possibility that such a relationship also exists between the surface molecules involved in signaling to immature thymocytes. Experiments designed to characterize the interrelationships between CD45RA and the CD2/CD28 molecules are presented in Chapter 6.

Soluble anti-CD45RA mAb does not by itself stimulate CD3$^-$4$^-$8$^-$ thymocytes; this is not surprising since cross-linking of cell surface molecules is a common prerequisite for cell signaling. In one experiment, an antibody against a CD45 common determinant was not only unable to stimulate CD3$^-$4$^-$8$^-$ thymocytes, its presence was actually inhibitory to anti-CD45RA effects. Inhibition may have been due to negative signalling, cancellation of positive signals, or simply to steric competition between the antibodies for different but spatially related epitopes. The latter seems unlikely as anti-CD45RA were present in about 3-fold excess over the antibody against CD45 common determinants. Inhibition by anti-CD45, under conditions in which anti-CD45RA is stimulatory, is a phenomenon which recurs in experiments described in Chapter 6.

The 4-fold enhancement in IL-2 mRNA expression by stimulated CD3$^-$4$^-$8$^-$ CD45R0$^-$ thymocytes, compared to CD3$^-$4$^-$8$^-$ thymocytes, is provocative in view of the fact that only about 7% of cells were removed. It is probable that this results at least partially from the observed enrichment for cells expressing a high density of CD45RA.

However it seems unlikely that this could account for the considerable increase in IL-2 mRNA seen in this fraction. Instead, it is possible that the effect resulted from depletion of an inhibitory cell type and/or enrichment of a stimulatory cell type. Depletion of CD45R0$^+$ cells also produced a 2-fold enrichment of cells with no detectable CD45 expression. These CD45$^-$ thymus cells are probably not of bone-marrow origin, and may be stromal cells. It is possible that if stromal elements are involved, in an ancillary fashion, in the activation of CD3$^-$4$^-$8$^-$ CD45RA$^+$ cells , their increased numbers in the CD45R0-depleted subset may account for its enhanced response to anti-CD45RA. Others have documented the involvement of thymic stromal cells in thymocyte development (149,164). Indeed, it is possible that the natural ligand for CD45RA may be a cell-surface molecule present on thymic stromal cells.

The interaction of IL-2 with its receptor is the only signal known to drive mature T cells to divide; the signals that drive proliferation of thymocytes, however, are largely unknown. In human and murine systems a large proportion of CD4$^-$8$^-$ thymocytes are IL-2R$^+$ (155,162,165-167), but it has been difficult to establish the functional relevance of the IL-2/IL-2R pathway in the proliferation of thymocytes. Some investigators have found that unstimulated IL-2R$^+$ murine thymocytes neither proliferate to IL-2 (168) nor internalise IL-2 (169), suggesting that the IL-2 receptors expressed in immature thymocytes are functionally defective, perhaps lacking the p75 component of the receptor. Rothenberg et al. have shown that the majority of thymocytes, including the CD4$^+$8$^+$ subset and some CD4$^-$8$^-$ double negatives, are unable to express IL-2R following *in vitro* stimulation (170-172). The non-inducible double negatives are considered to be the immediate precursors of the CD4$^-$8$^+$ immature blasts and double positive cells, i.e. the proliferating cells in the thymus. In the mouse, surface IL-2R appear to be transiently expressed only on a subset of double negatives, thus it is difficult to reconcile the absence of IL-2R on the proliferating cells of the thymus with the conclusion that IL-2/IL-2R interaction may drive thymocyte mitogenesis. Nevertheless, Jenkinson et al. reported that anti-IL-2R antibody added to fetal thymus organ culture completely inhibited proliferation (173), and De la Hera et al. (155) showed that the early spontaneous proliferation of $CD3^-4^-8^-$ human thymocytes is inhibited by anti-IL-2R. Most recently, Tentori et al. have contributed significantly to the resolution of this question by successfully blocking the development of mature-type $CD4^+$ or $CD8^+$ thymocytes in newborn mice from mothers treated with mAb to the IL-2R (156).

The extent to which microenvironmental interactions underly thymocyte development is equally unclear. Thymocytes have been observed in direct contact with various types of thymic epithelial cells (174), but it is not known whether these cell associations are related to mechanisms of tolerance, selection, proliferation, or other functions. The stimulation of early thymocytes by mAb to CD45RA and the selective expression of CD45RA by this set of cells implies the existence of a ligand for CD45RA distinct from that for the low m.w. CD45R0 isoform. The physiological ligands for CD45 isoforms are unknown but could be cell-surface molecules on the same, or different cells. Data presented in this chapter suggest that signals delivered by the interaction of CD45RA and its ligand contribute to the activation and proliferation of early human thymocytes.

V. DEVELOPMENTAL STAGE OF CD3$^-$4$^-$8$^-$ CD45R0$^-$ THYMOCYTES

Maturation of thymocytes from the earliest precursor stage involves the rearrangement and expression of TCR genes. The two types of TCR, TCR-$\gamma\delta$ and TCR-$\alpha\beta$, appear in that order during ontogeny (175-180). All four of the TCR genes are composed of variable (V), joining (J), and constant (C) region segments, and the TCR-β and -δ genes have additional diversity (D) segments rearranging between V and J (181). The γ gene is the first to rearrange during ontogeny, and the earliest identifiable T cell precursors in the adult thymus express γ transcripts (177,182). Only a single 1.5 kb transcript has been described, in contrast to the α, β and δ genes which all produce truncated transcripts, lacking V regions, in addition to the mature forms (177). The β gene rearranges after γ during ontogeny, followed by α (177,179,180,182). The δ gene rearranges at about the same time as γ, and is deleted from rearranged $\alpha\beta$ cells, since it is located within the α gene, between the Vα and Jα segments (181,183).

The sequence of TCR rearrangements during fetal development is reflected among the earliest T cell precursors in the adult thymus (177,182,184). Thus, CD3$^-$4$^-$8$^-$ adult murine thymocytes analyzed as a population, have rearranged γ genes, and a proportion of them have rearranged β (184).

As described in Chapter 4, CD3$^-$4$^-$8$^-$ thymocytes depleted of CD45R0$^+$ cells are enriched for the ability to produce IL-2 mRNA in response to CD45RA mAb and suboptimal PHA/PMA. Our hypothesis predicts that CD45R0$^+$ CD3$^-$4$^-$8$^-$ thymocytes are those that are committed to cell death. Since selection based on TCR specificity cannot occur at this early stage of differentiation, cell death, if it occurs, must result from other factors, possibly including nonfunctional TCR rearrangements on both chromosomes (177). Therefore, depletion of CD45R0$^+$ cells may enrich the CD3$^-$4$^-$8$^-$

population for cells in a slightly earlier stage of differentiation. In order to test this prediction, and to further characterize the cell populations studied in Chapters 4 and 5, Northern analyses of TCR gene expression were performed on both populations isolated from the same thymus. Parallel Northern analysis was done on unfractionated thymocytes and PBMC for comparison.

RESULTS

CD3δ mRNA is expressed in both $CD3^-4^-8^-$ *and* $CD3^-4^-8^-$ $CD45R0^-$ *populations.* CD3δ mRNA is detected in equivalent amounts in freshly isolated cells of all 3 populations of thymocytes tested, as well as in PBMC (lanes 1, 8, 16 and 21, Fig. 5.1). In stimulated cells of all 4 sets, the level of CD3δ mRNA is increased, and a maximal level is reached using stimuli that are suboptimal for IL-2 mRNA (eg compare lanes 4 and 7, 10 and 15, 17 and 20, Fig. 5.1).

Mature TCR-β mRNA is undetectable in either $CD3^-4^-8^-$ *or* $CD3^-4^-8^-$ $CD45R0^-$ *population.* Whereas mature TCR-β mRNA is detected in PBMC and unfractionated thymocytes (lanes 1 and 21, Fig, 5.1) only the truncated form is expressed by the $CD3^-4^-8^-$ population, and only in barely detectable amounts (lane 8, Fig. 5.1). The $CD3^-4^-8^-$ $CD45R0^-$ population also expresses only barely detectable β transcripts (lane 21). Stimulation of unfractionated thymocytes increased the expression of mature TCR-β mRNA (lanes 3-5, Fig. 5.1) and higher levels are reached when larger doses of PHA/PMA are used (lanes 6 and 7, Fig. 5.1). Stimulation of both $CD3^-4^-8^-$ and $CD3^-4^-8^-$ $CD45R0^-$ populations increased expression of the truncated form of TCR-β mRNA, but did not appear to lead to the expression of mature TCR-β mRNA (lanes 11-15, and 17-20). Unexpectedly, when all three cell populations were cultured in the presence of anti-CD45RA alone, there was a significant increase in expression of TCR-

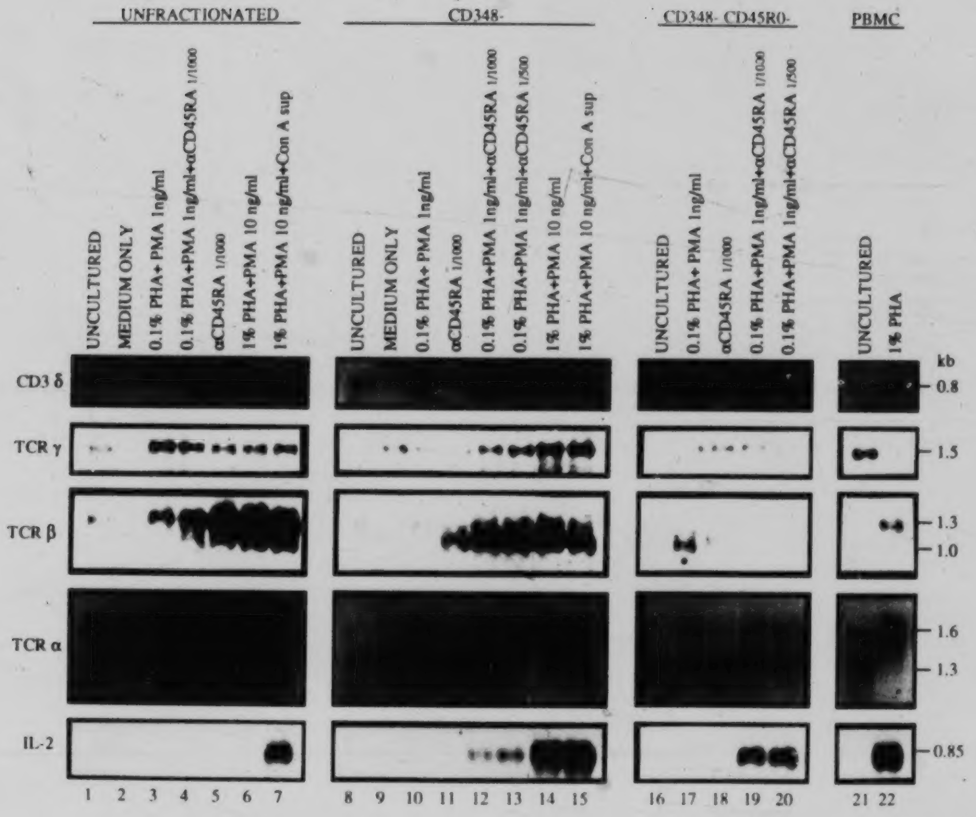

UNFRACTIONATED
CD348-
CD348- CD45R0-
PBMC
UNCULTURED
MEDIUM ONLY
0.1% PHA+ PMA 1ng/ml
0.1% PHA+PMA 1ng/ml+αCD45RA 1/1000
αCD45RA 1/1000
1% PHA+PMA 10 ng/ml
1% PHA+PMA 10 ng/ml+Con A sup
UNCULTURED
MEDIUM ONLY
0.1% PHA+ PMA 1ng/ml
αCD45RA 1/1000
0.1% PHA+PMA 1ng/ml+αCD45RA 1/1000
0.1% PHA+PMA 1ng/ml+αCD45RA 1/500
1% PHA+PMA 10 ng/ml
1% PHA+PMA 10 ng/ml+Con A sup
UNCULTURED
0.1% PHA+ PMA 1ng/ml
αCD45RA 1/1000
0.1% PHA+PMA 1ng/ml+αCD45RA 1/1000
0.1% PHA+PMA 1ng/ml+αCD45RA 1/500
UNCULTURED
1% PHA
CD3 δ
TCR γ
TCR β
TCR α
IL-2
kb
0.8
1.5
1.3
1.0
1.6
1.3
0.85
1 2 3 4 5 6 7
8 9 10 11 12 13 14 15
16 17 18 19 20
21 22

Figure 5.1. Northern blots of total cytoplasmic RNA from unfractionated, CD3⁻4⁻8⁻ and CD3⁻4⁻8⁻ CD45R0⁻ thymocytes,and PBMC. RNA was prepared either from freshly isolated cells (lanes 1, 8, 16 and 21) or from cells cultured for 24 h under conditions indicated. The filters were first probed with IL-2 cDNA, then stripped and probed sequentially for CD3δ, TCRα, TCRβ and TCRγ mRNA, in that order. RNA from 2 x 10^6 cells was loaded in each lane. Ethidium bromide staining of the gel confirmed that equivalent amounts of RNA were loaded.

β mRNA. This is most obvious in unfractionated thymocytes (lane 5), but it is also evident in the $CD3^-4^-8^-$ and $CD3^-4^-8^-$ $CD45R0^-$ subsets (lanes 11 and 18, respectively, Fig. 5.1). This observation is not likely to be artefactual, as similar patterns are not seen on probing the same filter for TCR-α (Fig. 5.1). This demonstrates that CD45 molecules transmit signals in the absence of other stimuli, and links those signals to regulation of TCR-β expression.

The level of TCR-β transcripts is elevated in unfractionated thymocytes compared to PBMC (Fig. 5.1), as previously reported (181).

Mature TCR-α mRNA is undetectable in freshly isolated $CD3^-4^-8^-$ or $CD3^-4^-8^-$ $CD45R0^-$ cells. Whereas mature TCR-α mRNA is easily detected in unfractionated thymocytes (lane 1, Fig. 5.1), there is none in either the $CD3^-4^-8^-$ or the $CD3^-4^-8^-$ $CD45R0^-$ population (lanes 8 and 16, Fig. 5.1). Interestingly, and in contrast to TCR-β mRNA, stimulated cells of both $CD3^-4^-8^-$ and $CD3^-4^-8^-$ $CD45R0^-$ subsets express low but detectable levels of mature TCR-α mRNA (lanes 10, 12-15, and 17,19 ,20), suggesting that a proportion of those subsets have functionally rearranged TCR-α genes. These results differ from those of Kinnon et al. (185) who found that TCR-α mRNA expression was not induced in stimulated murine $CD4^-8^-$ thymocytes. The difference may lie in the stimulation protocol used because the immature murine cells were stimulated by the receptor-independent combination of PMA and Ca^{2+} ionophore.

$CD3^-4^-8^-$ $CD45R0^-$ thymocytes express lower levels of TCR-α and -β transcripts than $CD3^-4^-8^-$ thymocytes. While freshly isolated cells of the $CD3^-4^-8^-$ and $CD3^-4^-8^-$ $CD45R0^-$ populations do not express mature forms of TCR-α and -β mRNA, a comparison of their relative degrees of differentiation can be made by measuring the

levels of truncated TCR-α and -β transcripts. The CD3$^-$4$^-$8$^-$ CD45R0$^-$ subset produced TCR-α and -β mRNA at approximately 85% of that produced by CD3$^-$4$^-$8$^-$ cells, as estimated by scanning densitometry of the autoradiograms.

Analysis of TCR-γ mRNA. Relatively high levels of TCR-γ mRNA were expressed by freshly isolated PBMC (lane 8, Fig. 5.1) and unfractionated thymocytes (lane 1). However, TCR-γ mRNA disappeared after stimulation of PBMC, but increased somewhat after stimulation of thymocytes (lanes 21 and 3-7). Similar to CD3-δ mRNA, the levels of expression after stimulation do not vary much with the doses of PHA and PMA used.

Freshly isolated CD3$^-$4$^-$8$^-$ thymocytes express only barely detectable levels of TCR-γ mRNA (lane 8, Fig. 5.1), and γ transcripts cannot be detected at all in freshly isolated CD3$^-$4$^-$8$^-$ CD45R0$^-$ cells (lane 16). Both populations increase production of γ mRNA after stimulation, but stimulated CD45R0-depleted CD3$^-$4$^-$8$^-$ cells express less than stimulated CD3$^-$4$^-$8$^-$ cells (lanes 17-20 compared to lanes 10-13, Fig. 5.1). These results were unexpected since immature murine thymocytes are known to be enriched for cells expressing high levels of γ mRNA (177,182), and Toribio et al. have clearly shown that human CD3$^-$4$^-$8$^-$ thymocytes express high levels of γ (186). An explanation is possibly provided by Kinnon et al. (185), who have described differential regulation of TCR-γ genes using Cγ1 versus Cγ2. Using RNA probe protection analysis to detect and quantitate mRNA transcripts it was demonstrated that Cγ1 is expressed in CD4$^-$8$^-$ thymocytes at very low levels (0.5 copies/cell) while Cγ2 is expressed at high levels (12 copies/cell). In addition, stimulation of CD4$^-$8$^-$ thymocytes with PMA and Ca^{2+} ionophore increased Cγ1 expression 4-fold, while Cγ2 expression decreases. Therefore, since the TCR-γ mRNA detected in our experiment (Fig. 5.1) was expressed at a lower level in immature thymocytes than in PBMC or unfractionated thymocytes, and increased following in vitro stimulation, it seems likely that Cγ1 is being detected to the exclusion of Cγ2. The cDNA probe used (Table 2.3) does, in fact, encode Cγ1; however, since there is high homology between Cγ1 and Cγ2 it was expected to hybridize with all TCR-γ mRNAs.

CD3$^-$4$^-$8$^-$ thymocytes are unresponsive to immobilized CD3 antibody. In order to ensure that the isolated thymocyte populations were not contaminated with CD3$^+$ peripheral blood cells at levels that are undetectable by IF or by Southern analysis, proliferation assays were performed using CD3 mAb immobilized on plastic microtiter plates. This assay is a very sensitive indicator of the presence of mature CD3$^+$ cells. Table 5.1 shows the results of three experiments demonstrating the lack of response of CD3$^-$4$^-$8$^-$ thymocytes to immobilized anti-CD3, confirming their immature phenotype and the lack of contamination by mature T cells, an important control both for the IL-2 mRNA induction experiments of Chapter 4 and the proliferation assays to be described in the next chapter.

SUMMARY

Molecular analysis of CD3$^-$4$^-$8$^-$ populations, isolated by antibody depletion from human thymus, has confirmed their early stage of differentiation. No mature TCR-β or -α transcripts can be detected in freshly isolated cells. Truncated β transcripts are just detectable in the CD3$^-$4$^-$8$^-$ population and CD3$^-$4$^-$8$^-$ CD45R0$^-$ subsets. After 24 hours of in vitro stimulation, there is an increase in truncated β message in both sets, but the level is lower in the CD45R0-depleted cells. Similarly, the expression of mature α transcripts that follows stimulation in vitro, is greater in the undepleted CD3$^-$4$^-$8$^-$ subset. By these criteria, CD3$^-$4$^-$8$^-$ thymocytes that have been depleted of CD45R0$^+$

TABLE 5.1

CD3⁻4⁻8⁻ thymocytes are unresponsive to αCD3

		cpm[a]	
		-	αCD3[b]
PBMC	Experiment 1	68	22444
	Experiment 2	306	35236
	Experiment 3	535	133817
CD3⁻4⁻8⁻ thymocytes	Experiment 1[c]	584	746
	Experiment 2	8659	7065
	Experiment 3	4567	4253

[a] Proliferation of CD3⁻4⁻8⁻ thymocytes was measured by uptake of [^{3}H]thymidine during the last 16 h of 4 or 5 day cultures. Proliferation of PBMC was measured at 3 days after a 6 h pulse. Cells were cultured in triplicate or quadruplicate, at 10^5/ml (PBMC) or 2×10^5/ml (thymocytes). Standard errors were <15% of the mean.

[b] Anti-CD3 mAb G19.4 was immobilized by precoating the wells of microtiter plates at 10 μg/ml.

c Data is taken from experiments in which the CD3-4-8- thymocytes proliferated well to alternate activation pathways (Chapter 6) cells are even less mature than undepleted CD3$^-$4$^-$8$^-$ cells.The relative immaturity of the CD3$^-$4$^-$8$^-$ CD45R0$^-$ subset substantiates the hypothesis that the earliest T-cell precursors are CD45RA$^+$.

Some of these results contrast with those of Toribio et al. who have reported that a proportion of CD2$^+$3$^-$1$^-$4$^-$8$^-$ "prothymocytes" have rearranged TCR-β genes and express mature β transcripts (186,187). We believe that the inconsistency is due to differences in cell preparation techniques. In our hands, and as reported by others (158,188), the high degree of purity that can be acheived either by cell sorting or by depletion with immunomagnetic beads, is unattainable using antibody and complement depletion.

VI. DIFFERENTIAL MODULATION OF CD3$^-$4$^-$8$^-$ THYMOCYTE PROLIFERATION BY ANTI-CD45RA AND ANTI-CD45

Signals transmitted via CD45RA restored the ability of suboptimally stimulated CD3$^-$4$^-$8$^-$ thymocytes to induce expression of IL-2 and IL-2R mRNA (27, Chapter 4). However, CD45RA antibodies were by themselves unable to activate the cells. The additional requirement for suboptimal doses of PHA and PMA suggested that signaling molecules other than CD45RA were also involved in the activation of CD3$^-$4$^-$8$^-$ thymocytes. Therefore we sought to explore the additional signaling requirements of T cell progenitors, and the interplay between CD45RA and other potential signaling molecules known to be present on the surfaces of these cells.

Over 15 distinct cell-surface molecules reportedly have activation properties on T cells (189). The best characterized of these are the TCR/CD3 complex, CD2 and CD28. CD3 is a group of five TCR-associated molecules thought to transduce signals received by the TCR following antigen/ MHC binding (190). CD2 is a 50 kDa transmembrane glycoprotein expressed on all T cells and over 95% of thymocytes (191-193). Three epitopes in the external region of CD2 are important in triggering activation (191), and antibodies against any pair of epitopes are necessary and sufficient to activate CD3$^+$ T cells (118). CD28 is a 44kDa glycoprotein with homology to the immunoglobin gene superfamily (194), expressed on 80% of peripheral blood T cells (195), and 10-20% of thymocytes, predominantly of mature phenotype (162). The activation pathway of CD28 differs fundamentally from that of CD2 and CD3 (196-198): It is resistant to the inhibitory effects of cyclosporin (148,199-201), stabilizes IL-2 and certain other cytokine mRNAs (202), and results in increased cGMP (203). CD28 antibodies substantially augment proliferative responses of mature T cells to anti-CD3 and anti-CD2 (204).

The majority of CD3$^-$4$^-$8$^-$ thymocytes express CD2 (Fig. 4.1) and CD45RA (9,10 and Fig. 4.1), and 14-20% express a low density of CD28 (Pilarski, unpublished data). It is entirely possible that a larger number express an undetectable level of CD28, since the density of CD28 on unstimulated peripheral T cells is also very low, and a clearly negative population is ill-defined. Immunohistological staining of thymus sections shows that CD28 is expressed predominantly in the medulla, with scattered positive cells in cortical areas (Gillitzer,R. and Pilarski,L.M., unpublished data). Currently there is very little published data on specific activation properties of immature thymocytes: Denning et al. have shown that mitogenic CD2 antibodies induce proliferation of CD3$^-$4$^-$8$^-$ thymocytes in the presence of IL-2 or PMA (158), and Toribio et al. reported that CD3$^-$4$^-$6$^-$8$^-$ thymocytes proliferate in response to either CD2 or CD28 even without additional factors (157).

In this study, we chose to focus on the role of three cell surface molecules, CD2, CD28 and CD45, all of which have well-characterized signaling ability in mature T cells, in inducing and/or modifying proliferative responses of CD3$^-$4$^-$8$^-$ thymocytes. The data described here confirms that CD2 and CD28 antibodies induce proliferation of CD3$^-$4$^-$8$^-$ thymocytes, although we find that significant proliferation is only obtained in the presence of both sets of antibodies, and only in the presence of additional factors such as either PMA or IL-7. IL-7 was originally isolated from a bone-marrow stromal cell line and shown to be active in B cell growth (205). IL-7 mRNA is expressed at a high level in the murine thymus (205), and has been shown to participate in thymocyte proliferation (206-207), as well as in the growth of mature T cells (208).

Anti-CD45RA mAb do not synergize with suboptimal PHA to induce proliferation despite the evidence that IL-2 mRNA is expressed. However, antibodies to CD45RA or to common determinants of CD45 have substantial and different modulatory effects when coupled to anti-CD28. The data suggests that proliferation of T cell progenitors is accomplished through an orchestrated series of signaling events, probably reflecting sensitivity to the cellular microenvironment.

RESULTS

Proliferation of CD3$^-$4$^-$8$^-$ thymocytes. In a pilot experiment, a large panel of antibodies and antibody homo- and hetero-conjugates was screened for the ability to promote proliferation of CD3$^-$4$^-$8$^-$ thymocytes in the absence or presence of PMA (Table 6.1). The conjugates, donated by Dr.Ledbetter, consisted of covalently-linked mAb prepared as described (99,102). Heteroconjugates were prepared at a 1:1 molar ratio using a technique which ensured that antibody molecules of one specificity could only be conjugated to antibody molecules of the second specificity (99).

Two main points can be made from the results of this experiment. First, mAb against CD45RA was unable to augment proliferation induced by a suboptimal amount of PHA and PMA, in contrast to our observation of enhanced IL-2 and IL-2R mRNA expression under identical conditions (Chapter 4, 27). Second, the only set of cultures that proliferated, excluding those containing PHA, was one that contained both CD2 and CD28 antibodies in the presence of PMA and a cross-linking antibody, 187.1. Ledbetter et al. have shown that mAb against one epitope of CD2 will induce proliferation of PBMC in the presence of IL-2, PMA or CD28 mAb, if the CD2 mAb is cross-linked either using a second reagent antibody, or in the form of covalently

TABLE 6.1

Proliferation of $CD3^-4^-8^-$ thymocytes

Stimulus	-PMA	+PMA[a]
-	2337[b]	2623
0.5% PHA	25714	24588
0.1% PHA	5661	5062
0.1% PHA + 3AC5[c]	3689	5758
9.6 (CD2)	1638	1435
9.3 (CD28)	2047	1798
9.4 (CD45)	2046	2113
3AC5 (CD45R)	1905	2358
2.1 +2.9 (CD2)	1947	1715
2.1 + 2.9 +3AC5	2032	2757
2.1 + 2.9 +9.4	1883	2339
9.6x9.6	1169	1099
9.3x9.3	1081	1877
9.4x9.4	1089	1371
3AC5x3AC5	1478	2080
G19.4xG19.4 (CD3)	1791	2594
9.6x9.4	2550	2247
9.6x3AC5	1947	1841
9.3x9.4	1431	2741
9.3x3AC5	2014	1992
G19.4xG19.4	1519	1552
G19.4x3AC5	1132	2487
9.4x3AC5	1278	1350
9.6 + 9.3	1577	1555
9.6 + 9.3 + 187.1	2241	5038
9.6 + 3AC5	1864	1614
9.6 + 3AC5 + 187.1	1273	2432
3AC5 + 187.1	1549	1559

[a] PMA 1 ng/ml; [b] cpm/$2x10^5$ cells, mean of triplicate cultures, measured at 5 days; [c] all mAb were used at 1 µg/ml, except 187.1 which was used at 4x the concentration of other mAb present. Specificities are shown in parentheses. 2.1 and 2.9 are a mitogenic pair of anti-CD2 mAb, recognizing 2 distinct epitopes.

coupled homoconjugates (101). However in this experiment , the presence of both PMA and CD28 mAb was required. A mitogenic pair of CD2 mAb was also insufficient to induce proliferation, either alone or in combination with CD45RA mAb. In general, the results suggest that progenitor thymocytes respond weakly to stimulation via CD2 and CD28, and are less sensitive than mature T cells, as measured by proliferation.

Comparison of PMA, IL-2 and IL-7 as costimulators of CD3$^-$4$^-$8$^-$ thymocyte proliferation. In the experiment of Table 6.1, only minimal proliferation was achieved using CD2, CD28, 187.1 anti-mouse Ig, and PMA (Proliferation Index [PI] = 1.9; PI = mean uptake of 3H-TdR in the antibody-stimulated cells divided by the mean value in anibody-unstimulated cells). In all of the experiments following, a mitogenic pair of CD2 mAb was used in place of the single CD2 antibody and 187.1, as this resulted in a marked increase in PI (Table 6.2 and Fig. 6.1). In addition, the effect of adding IL-2 or IL-7, at various concentrations, was tested in 3 separate experiments (Table 6.2). It was found that CD3$^-$4$^-$8$^-$ thymocytes proliferated in the presence of IL-2 without any additional stimulation, and the enhanced proliferation in the presence of CD2 and CD28 mAb was minimal (PI $\leq$ 2.4 at all concentrations tested, Table 6.2). This effect was observed in 4/4 experiments, and is consistent with data reported by Denning et al. (158) and Toribio et al. (157). IL-2 was therefore not used in further experiments as a co-stimulator for antibody-induced proliferation; however, it is worth noting that the observation implies that human CD3$^-$4$^-$8$^-$ thymocytes do bear IL-2R (3,209,210), despite the inability of some investigators to detect them (211). This may be due to technical difficulties with some anti-IL-2R mAb, such as anti-Tac, in detecting IL-2R that have bound IL-2. In the murine thymus approximately 50% of CD4$^-$8$^-$ thymocytes

TABLE 6.2

Comparison of PMA, IL-2 and IL-7 as costimulators of $CD3^-4^-8^-$ thymocyte proliferation

	Experiment 1		Experiment 2			Experiment 3	
Stimulus	-	2.1+2.9+9.3[a]	-	2.1+2.9+9.3	2.1+2.9+(9.3x9.3)	-	2.1+2.9+(9.3x9.3)
-	451[b]	459 (1.0)[c]	434	491 (1.1)	626 (1.4)	1553	14320 (9.2)
PMA[d]	1602	19053 (11.9)	329	474 (1.4)	2961 (9.0)	2341	18366 (7.8)
IL-2 1[e]	1479	1762 (1.2)					
IL-2 10	2223	4619 (2.1)	7774	13227 (1.7)	18489 (2.4)	31323	43791 (1.4)
IL-2 25	4043	6376 (1.6)					
IL-7 1[e]	289	347 (1.2)					
IL-7 10	245	345 (1.4)					
IL-7 25	293	335 (1.1)					
IL-7 100			538	2801 (5.2)	4567 (8.5)	2346	22892 (9.8)
IL-7 300			1319	2592 (2.0)	7309 (5.5)		
IL-7 1000			4453	9282 (2.1)	12657 (2.8)		

[a] antibodies were used at 1µg/ml. 2.1 and 2.9 are a mitogenic pair of anti-CD2 mAb, recognizing 2 distinct epitopes; 9.3 = CD28; (9.3x9.3) = CD28 conjugate; [b] cpm/$2x10^5$ cells, mean of triplicate cultures, measured at 5 days.

[c] Proliferation Index = mean uptake of ^{3}H-TdR in the antibody-stimulated cultures divided by the mean value in antibody-unstimulated cultures; [d] PMA at 1 ng/ml; [e] Units/ml.

bear detectable IL-2R (170); however, in contrast to the equivalent human cells, they do not proliferate to IL-2 without additional stimulus (168,169).

CD3$^-$4$^-$8$^-$ thymocytes also proliferated to IL-7, without additional stimulus at concentrations in excess of 100 U/ml (Table 6.2). However, 100 U/ml IL-7 proved to be a suboptimal concentration which synergized well with CD2 and CD28 mAb in inducing proliferation of early thymocytes (Table 6.2, PI = 5.2 and 9.8, in 2 separate experiments).

Some variability among individuals was noted. For example, in experiment 2 of Table 6.2, the cells did not proliferate to CD2 and CD28 in the presence of PMA, although they did in the presence of IL-7. In the same experiment, the cells did proliferate in response to CD2 and the CD28 homoconjugate in the presence of PMA. Variability in cellular proliferation between individuals is not unexpected, and has been reported previously in studies on mature human T cells (28). Perhaps this is particularly true in experiments using cells from thymi derived from children of various ages, and suffering variable degrees and types of physiological stress. Nevertheless, the consistent observation was that the combination of CD2 mAb and CD28 homoconjugate caused significant proliferation of CD3$^-$4$^-$8$^-$ thymocytes, in the presence of PMA. Occasionally these mAb caused proliferation even in the absence of PMA, as in experiment 3 of Table 6.2, but this was the exception. Whenever CD28 mAb was used in the same experiment in both unconjugated and conjugated forms, the latter was always a more potent stimulator of proliferation, suggesting a requirement for cross-linking of CD28.

Synergy between CD2 and CD28 mAb in inducing proliferative responses. A mitogenic pair of CD2 mAb induced only a minimal degree of proliferation in the absence of CD28 mAb (Fig. 6.1; PI ≤ 2.0), and CD28 mAb alone had no proliferative influence at all, regardless of the presence of IL-2, PMA (Fig. 6.1) or IL-7 (not shown). This was the case in all of 4 experiments. However, there was a strong response to CD2 and CD28 mAb when tested together in the presence of PMA (Fig. 6.1), or IL-7 (not shown). Anti-CD45RA also induced a minimal proliferative response (Fig.6.1), and this effect was additive in combination with CD2 mAb, a consistent observation in 3/4 experiments. CD28 and CD45RA antibodies together were not stimulatory (Fig. 6.1).

Inhibition by anti-CD45. Figure 6.2 shows that conjugation of CD45RA mAb to CD28 mAb either augments or has no effect on proliferation induced by CD2 and CD28. In contrast, complete inhibition occurs when mAb to a CD45 common determinant (9.4) is conjugated to the CD28 mAb. Figure 6.3 shows the results of a separate experiment that confirms and extends these observations. In most experiments, a combination of CD2 and CD28 homoconjugate (9.3x9.3) provided maximal stimulation of $CD3^-4^-8^-$ thymocytes. Figure 6.3 also shows a marked difference between CD45RA and CD45 homoconjugates when they are added to CD2-stimulated cultures either in the presence or absence of PMA. Overall, the results show that the effect of the CD45RA-CD28 heteroconjugate on CD2-induced proliferation is either stimulatory or negligible, whereas the effect of CD45-CD28 is always inhibitory.

Physical association of CD45 with CD28 is required for inhibition. The effect of the heteroconjugates is to force 2 distinct molecules into proximity on the cell surface. It is possible that such an association may be induced in the physiological environment by

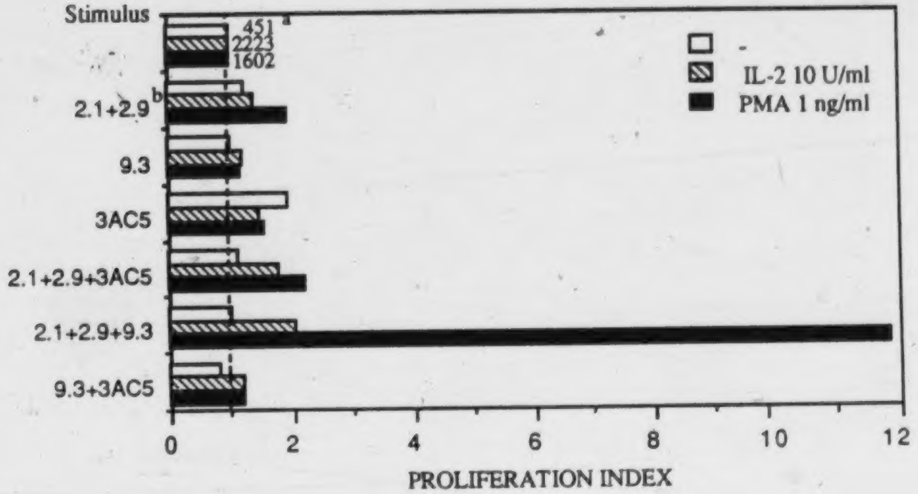


Figure 6.1. Anti-CD28 synergizes with anti-CD2 to induce proliferation of CD3$^-$4$^-$8$^-$ thymocytes in the presence of PMA. CD3$^-$4$^-$8$^-$ thymocytes were cultured in triplicate at 10^6/ml for 5 days in conditions indicated. Proliferation Index = mean uptake of ^{3}H in the antibody-stimulated cultures divided by the mean value in the antibody-unstimulated cultures (dotted line = PI 1.0).

[a] mean cpm of antibody-unstimulated cultures. [b] antibodies used at 1 µg/ml; 2.1 and 2.9 are a mitogenic pair of anti-CD2 mAb, recognizing 2 distinct epitopes; 9.3 = CD28, 3AC5 = CD45RA.

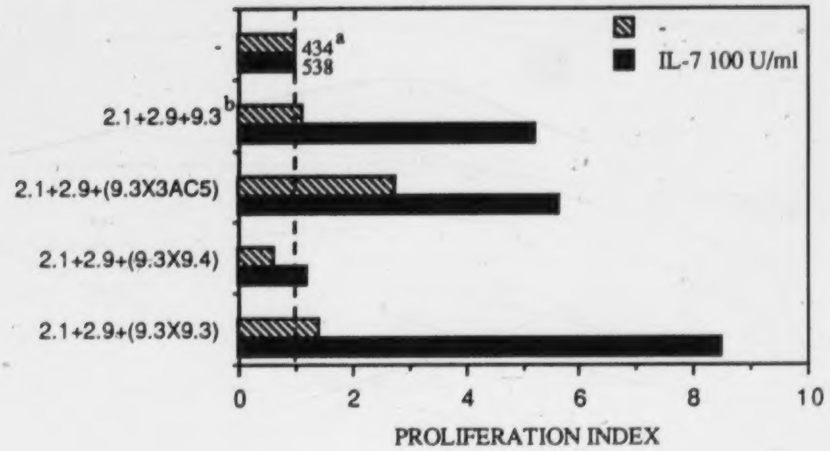


Figure 6.2. Anti-CD45 inhibits proliferation induced by CD2 and CD28 mAb. $CD3^-4^-8^-$ thymocytes were cultured in triplicate at 10^6/ml for 5 days in conditions indicated. Proliferation Index = mean uptake of ^3H in the antibody-stimulated cultures divided by the mean value in the antibody-unstimulated cultures (dotted line = PI 1.0).

[a] mean cpm of antibody-unstimulated cultures.[b] antibodies used at 1 µg/ml; 2.1 and 2.9 are a mitogenic pair of anti-CD2 mAb, recognizing different epitopes; 9.3 = CD28, 3AC5 = CD45RA, 9.4 = CD45. Conjugates indicated in parentheses.

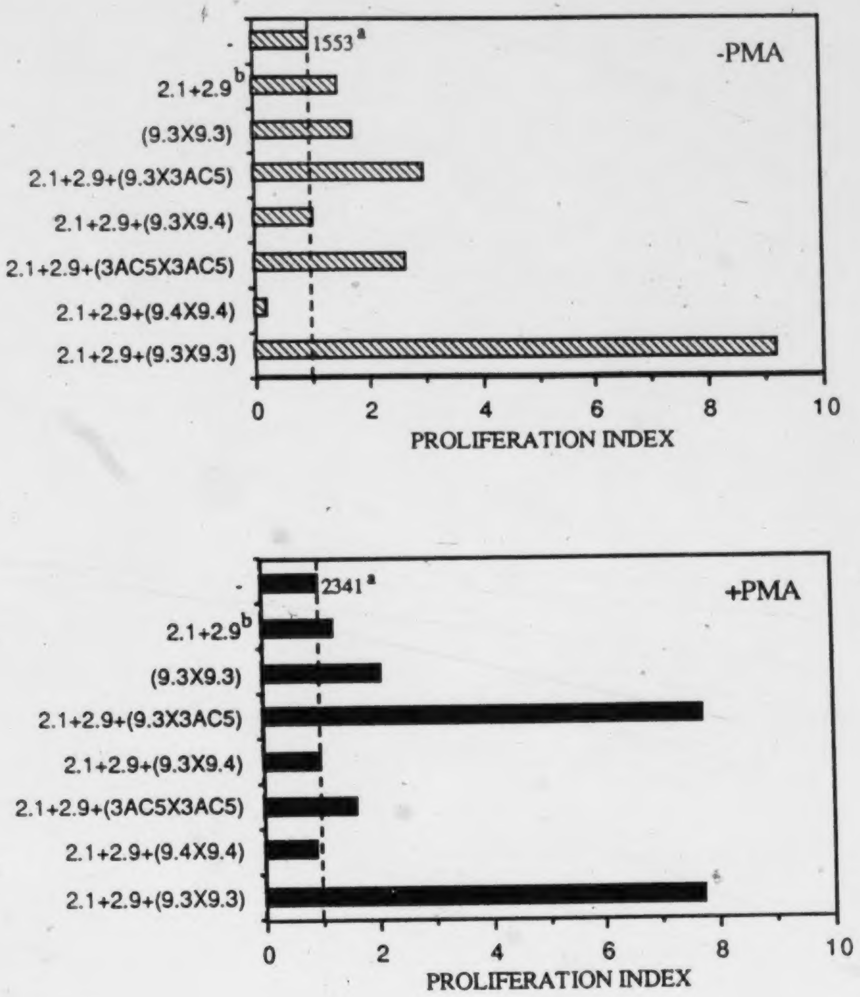


Figure 6.3. Differential modulation of CD2 plus CD28-induced proliferation by anti-CD45and anti-CD45RA. CD3$^-$4$^-$8$^-$ thymocytes were cultured in triplicate at 10^6/ml for 5 days in conditions indicated. Proliferation Index = mean uptake of ^{3}H in the antibody-stimulated cultures divided by the mean value in the antibody-unstimulated cultures (dotted line = PI 1.0). [a] mean cpm of antibody-unstimulated cultures. [b] antibodies used at 1 μg/ml; 2.1 and 2.9 are a mitogenic pair of anti-CD2 mAb, recognizing different epitopes; 9.3 = CD28, 3AC5 = CD45RA, 9.4 = CD45. Conjugates indicated in parentheses.

binding to the appropriate ligands, although this of course remains to be demonstrated. In order to determine if the effects noted above require the physical association of CD28 with CD45, the antibodies were added either singly, or as heteroconjugates (Table 6.3). Interestingly, we found that there was a synergistic effect when CD45RA mAb was added separately from anti-CD28, and this combination of stimuli allowed the cells to proliferate beyond the maximal amount seen previously with CD2 and CD28 homoconjugate (PI = 11.64 v. 7.6 in this expt., Table 6.3). On the other hand, when the 9.4 mAb to CD45 common determinants was added separately from anti-CD28 there was no effect on proliferation, but the heteroconjugate was completely inhibitory, as observed previously.

These results are similar in some respects to analagous experiments using PBMC (99), in which a) proliferation induced by anti-CD3 was inhibited by immobilized, but not soluble, CD45 antibodies and b) proliferation induced by CD2 or CD28 was inhibited by anti-CD45 in the presence of a cross-linking second antibody, but enhanced in its absence (99). Thus, the inhibitory effect of anti-CD45 mAb, with the requirement for cross-linking, is a common feature of both mature T cells and their progenitors.

Immobilized CD45RA mAb enhances stimulation by CD2 and CD28. It was observed by Ledbetter et al. (99) that immobilized antibodies against either CD45RA or CD45 common determinants inhibited the CD3 response. Since no inhibitory effect of CD45RA mAb on $CD3^-4^-8^-$ thymocytes had been observed under the conditions tested thus far, we wished to examine the effect of immobilized CD45 mAb in our system. Therefore, in the experiment presented in Table 6.4, CD45 mAb were added to the cultures either in soluble form, or immobilized on plastic by precoating the wells of the

TABLE 6.3

CD45 inhibits proliferation of $CD3^-4^-8^-$ thymocytes by physical association with CD28

Stimulus	cpm[a]	PI[b]
-	1176	
9.1+9.6[c]	1367	1.16
9.1+9.6+9.3	6125	5.21
9.1+9.6+3AC5	2232	1.90
9.1+9.6+9.3+3AC5	13683	11.64
9.1+9.6+(9.3x3AC5)	8191	6.97
9.1+9.6+9.3+9.4	9416	8.01
9.1+9.6+(9.3x9.4)	1404	1.19
9.1+9.6+(9.3x9.3)	8940	7.60
9.1+9.6+(3AC5x3AC5)	1809	1.54
9.1+9.6+(9.4x9.4)	1109	0.94
9.3+3AC5	761	0.65
9.3x3AC5	1355	1.15
9.3+9.4	937	0.80
9.3x9.4	1076	0.92

[a] cpm/2x10^5 cells, mean of quadruplicate cultures, measured at 4 days; [b] Proliferation Index = mean uptake of ^{3}H-TdR in the antibody-stimulated cultures divided by the mean value in antibody-unstimulated cultures; [c] antibodies were added at 1 µg/ml; 9.1+9.6 is a mitogenic pair of anti-CD2 mAb, equivalent to 2.1 and 2.9 used in previous experiments, 9.3 = CD28, 3AC5 = CD45RA, 9.4 = CD45. PMA was added to all cultures at 1 ng/ml.

TABLE 6.4

CD45 modulates stimulation by CD2 and CD28

	$CD3^-4^-8^-$ thymocytes		PBMC
Stimulus^a	-PMA	+PMA^b	+PMA^b
3AC5 (immobilized)	1.94^c	0.98^c	0.96^c
9.4 (immobilized)	2.44	0.87	0.92
9.1+9.6	1.55	7.50	9.49
9.1+9.6+3AC5 (immobilized)	5.72	7.18	22.11
9.1+9.6+9.4 (immobilized)	2.69	8.14	10.27
9.1+9.6+9.3	1.33	35.64	17.93
9.1+9.6+9.3+3AC5 (immobilized)	5.44	48.76	24.21
9.1+9.6+9.3+3AC5 (soluble)	1.82	48.45	27.22
9.1+9.6+(9.3x3AC5)	1.26	52.29	26.52
9.1+9.6+9.3+9.4 (immobilized)	3.45	25.70	6.28
9.1+9.6+9.3+9.4 (soluble)	1.18	32.29	28.34
9.1+9.6+(9.3x9.4)	1.36	12.89	22.99

Proliferation of $CD3^-4^-8^-$ thymocytes was measured by uptake of [^{3}H]thymidine during the last 16 hr of a 4-day culture period. Proliferation of PBMC was measured at 3 days after a 6 h pulse. All cells were cultured in quadruplicate at 10^5/well.

[a] 9.1and 9.6 is a mitogenic pair of anti-CD2 mAb, equivalent to 2.1 and 2.9 used in previous experiments, 9.3 (CD28), 3AC5 (CD45RA) and 9.4 (CD45). All antibodies were added in solution at 1 μg/ml. 3AC5 and 9.4 were immobilized by precoating the wells at 10 μg/ml. [b] PMA was added at 1 ng/ml. [c] Proliferation Index = mean uptake of ^{3}H-TdR in the antibody-stimulated cultures divided by the mean value in antibody-unstimulated cultures. Mean cpm of antibody-unstimulated cultures = 584 without PMA and 1214 with PMA for thymocytes; 3506 for PBMC. Standard errors were <20% of the mean for the majority of groups.Individual cpm in those groups with higher standard errors did not overlap with cpm in the relevant control groups.

microtiter plates. Duplicate sets were cultured in the presence or absence of PMA. In addition, a parallel assay was performed using PBMC.

In the absence of PMA a definite enhancing effect of immobilized CD45 mAb is observed, both with CD45RA mAb and with antibodies to CD45 common determinants, although the effect is more pronounced with the CD45RA antibody (Table 6.4). Enhancement of CD2-induced proliferation is observed even in the absence of CD28, and is not obtained when the CD45 antibodies are present in soluble form. In the presence of PMA, anti-CD45RA augmented CD2 plus CD28 induced proliferation, whether it was present in soluble form, or immobilized (Table 6.4). Under the same conditions antibodies to the CD45 common determinant had no effect when it was present in soluble form, but inhibited CD2 plus CD28 induced proliferation by 28% when it was immobilized, and by 64% when it was covalently coupled to CD28, as previously observed.

There is a possibility that when the anti-CD45 mAb 9.4 is covalently linked to anti-CD28, it interferes with the ability of anti-CD28 to bind to its target. The apparent inhibition by 9.4 would then be actually due to a lack of CD28 stimulation. However, the CD45xCD28 heteroconjugate does not inhibit proliferation of PBMC as it does proliferation of $CD3^-4^-8^-$ thymocytes (Table 6.4), providing an important control for the ability of the CD45xCD28 heteroconjugate to bind to CD28 on the cell surface.The effects of CD45RA antibodies, and antibodies against CD45 common determinants, on CD2/28-induced proliferation of PBMC are otherwise quite similar to the effects observed on $CD3^-4^-8^-$ thymocytes (Table 6.4).

DISCUSSION

The in vitro proliferative capacity of CD3$^-$4$^-$8$^-$ thymocytes and modulatory effects of CD45 have been investigated. We have found that CD3$^-$4$^-$8$^-$ thymocytes proliferate in response to stimulation through CD2 and CD28. Occasionally proliferation occurred in response to CD2 without CD28, as reported by others (157,158), but this was the exception. IL-2 was found to be a potent growth factor for CD3$^-$4$^-$8$^-$ thymocytes to the extent that it was unsuitable as a costimulator in experiments designed to test antibody effects. IL-7 and PMA were both useful as costimulators, but since there seemed to be no advantage in using IL-7, ultimately PMA was used exclusively.

We find considerable quantitative variation between experiments that is apparently not due to experimental conditions, and we conclude that variation is most likely to be an expression of differences between individuals. Depletion of CD3$^+$, CD4$^+$, CD8$^+$ cells and B cells results in cell preparations enriched for non-lymphoid cells as well as T cell progenitors, but preparations from different individuals may vary in their content of accessory cells, and also in the relative degree of differentiation of the CD3$^-$4$^-$8$^-$ thymocyte population itself.

The stimulatory capacity of CD45RA mAb, and the inhibitory effect of the 9.4 anti-CD45 mAb when coupled to anti-CD28, was evident in all of 6 experiments. The stimulatory effect of anti-CD45RA may be the result of direct enhancement of IL-2 production, as suggested by the experiments described in chapter 4, or alternatively, may be due to blocking of an interaction that is otherwise inhibitory.

The inhibition effected by antibody to the CD45 common determinant when it is covalently linked to anti-CD28 is somewhat puzzling. One possibility is that the coupling of the two antibodies interferes with the ability of CD28 to bind to its ligand. Two points argue against this explanation: 1) the heteroconjugate composed of anti-CD45RA and anti-CD28 does not interfere with CD28 stimulation, and 2) the CD45xCD28 heteroconjugate does not inhibit proliferation of PBMC. Nevertheless, given that CD45-CD28 interactions on thymocytes are different than on PBMC, it would be useful to test this possibility by cross-linking CD45 and CD28 antibodies using anti-mouse Ig, or by immobilizing both on the same surface.

An alternative explanation might be that the CD45 and CD28 molecules are brought into proximity in different orientations by the two heteroconjugates: If tyrosine phosphorylation of CD28, or a closely associated molecule, is required for activation, then it is possible that crosslinking CD28 with CD45 may inhibit the CD28 activation pathway by inducing dephosphorylation via the PTPase activity of CD45. Dephosphorylation of CD28, or CD28-associated molecules, induced in this way could very well depend on the CD45 epitope involved in the ligation to CD28. The external domain of CD45 has an elongated, rod-shaped configuration, with the cytoplasmic domains positioned at one end of the rod (68). The location of the epitope recognized by the 9.4 mAb is likely to be at some distance from the CD45RA epitope which is known to be near the N-terminus of the extended "insert" portion of the molecule (71,72). Therefore, positioning of the cytoplasmic domains close to CD28 when ligated with antibody against one epitope of CD45 , but not another, seems entirely likely (Fig 6.4).

Differential modulation of $CD3^-4^-8^-$ thymocyte activation, by antibodies against different epitopes on the same set of CD45 molecules, suggests that ligands for different epitopes on CD45RA may control its activity in modulating responses. It

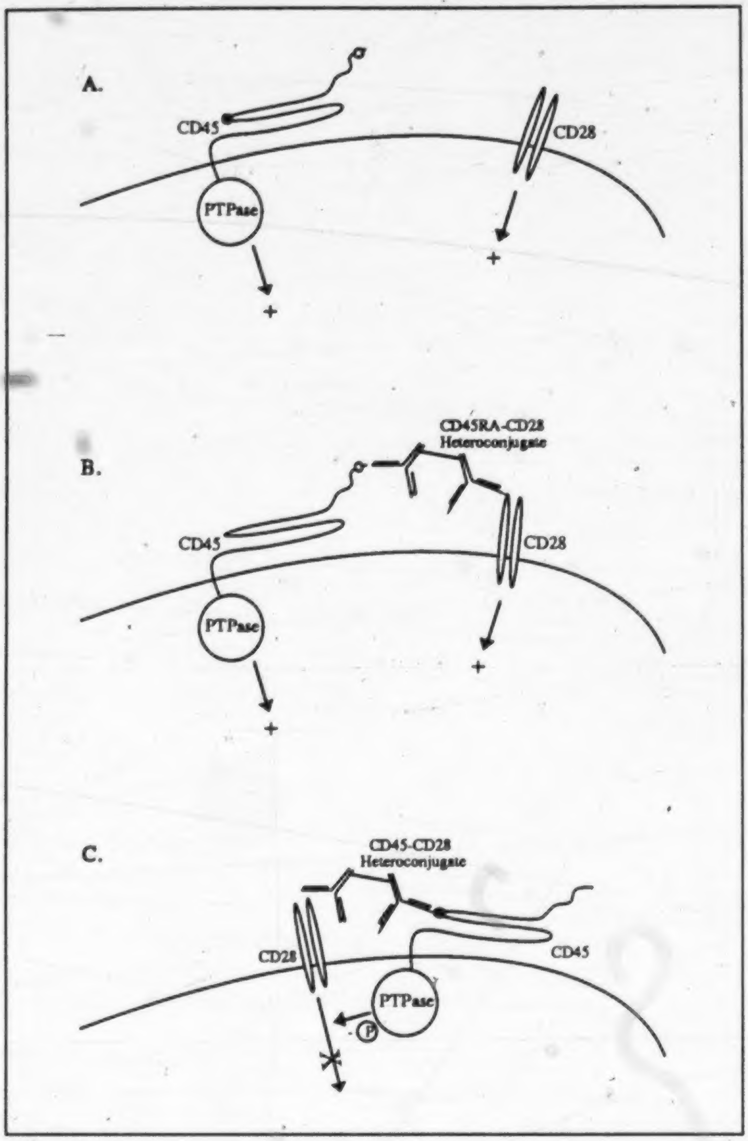

A.
CD45
CD28
PTPase
+
+
B.
CD45RA-CD28
Heteroconjugate
CD45
CD28
PTPase
+
+
C.
CD45-CD28
Heteroconjugate
CD28
CD45
PTPase
P

Figure 6.4. Possible mechanism of differential modulation of CD28-induced activation by heteroconjugates composed of anti-CD28 and either anti-CD45RA or antibodies against CD45 common determinants. In *A*, CD28 and CD45RA molecules transmit positive activation signals when stimulated by unlinked antibodies against CD28, CD45RA, or common determinants of CD45. Antibodies are not illustrated in A. Epitopes unique to CD45RA are located near the N-terminus (open circle).The exact location of the 9.4 common determinant (closed circle) and its spatial relationship to the CD45RA epitope are speculative. In *B*, the heteroconjugate composed of CD28 and CD45RA antibodies does not bring the PTPase cytoplasmic domain of CD45 into close proximity with CD28. In contrast, the CD28-CD45 heteroconjugate forces an association between the two molecules resulting in dephosphorylation of CD28 or CD28-associated molecules, and inhibition of activation (*C*).

remains to be determined whether or not these phenomenological observations reflect a physiological situation.

Although we had previously shown that antibodies against CD45RA, in combination with low doses of PHA and PMA, induced the expression of IL-2 and IL-2R mRNA in CD3$^-$4$^-$8$^-$ thymocytes, identical culture conditions did not allow cellular proliferation. In experiments not shown we found that the important co-factor for anti-CD45RA in IL-2 and IL-2R mRNA induction was PMA, not PHA. In some experiments CD45RA mAb did induce a small proliferative response, but this was more easily seen in the absence of PMA (see Fig. 6.1 and Table 6.4). Presumably the stimulus provided by activation through CD45RA is adequate to either initiate or augment IL-2 and IL-2R gene transcription, but insufficient for a proliferative response.

Maximal proliferation of CD3$^-$4$^-$8$^-$ thymocytes is obtained using combinations of CD2, CD28 and CD45RA antibodies. Studies using mature T cells have demonstrated that CD28-induced activation stabilizes IL-2 mRNA (202), allowing accumulations of IL-2 far beyond that needed for autocrine proliferation, and probably induces IL-2 gene transcription by a different pathway from that activated by CD2 (and CD3) (196-198). Control of proliferation in vivo conceivably might be at the level of CD28, in turn controlled by CD45 interactions. Activation of progenitor thymocytes via CD28 appears to delay, and possibly abrogate the down-regulation of CD45RA mRNA (Fig. 3.6, Chapter 3), suggesting a dependence on CD28 for the coexistence of CD45RA on the surface of activated cells. Perhaps stimulation of CD3$^-$4$^-$8$^-$ thymocytes through CD28 (and CD2) is the norm. As long as this continues, co-expression of CD45RA also continues. Stimulation through CD45RA, meanwhile, augments proliferation of the cells, perhaps via the dephosphorylation and consequent activation of substrates intermediate in the CD28 activation pathway. The appearance of CD3/TCR on the cell surface as early thymocytes differentiate, and subsequent activation via that complex, may uncouple the CD28/CD45 interaction allowing the cells to either switch to lower m.w. isoforms of CD45, or remain CD45RA$^+$, depending on the nature of the CD3 signals.

Inhibition caused by the CD45xCD28 heteroconjugate is difficult to interpret, however, the lack of inhibition of mature T cells under similar conditions suggests that mature and immature T cells are different with respect to their activation programs; more specifically, in the ways that some of the key surface triggering molecules interact. It will be necessary to identify the natural ligands for CD28 and the CD45 isoforms, as well as the substrates for the enzymatic activity in the cytoplasmic domains of CD45, before any real advance can be made in the understanding of how these molecules interact in the orchestration of T cell and early thymocyte activation.

VII. SUMMARY AND SYNTHESIS

Immune responses involve multi-faceted interactions between a variety of lymphoid and non-lymphoid cells, resulting in the selection and expansion of antigen-specific lymphocyte clones. After a primary antigenic challenge, antigen-inexperienced T cells not only proliferate, but also differentiate further into a set of memory and effector cells. In humans, antigen-inexperienced and memory T cells can be distinguished by cell surface phenotype, including differences in expression of CD45 isoforms. The shift from high to low m.w. isoforms of CD45 appears to represent a change of fundamental significance, either in the types of cellular interactions mediated by different CD45 isoforms (different ligands) in primary versus memory T cells, or in the nature of the signals transmitted via high and low m.w. isoforms.

As part of this project, the kinetics of the transition from high to low m.w. CD45 mRNA was investigated: We found that there is an immediate decrease in the level of high m.w. CD45 mRNA following mitogen stimulation of T cells, quickly followed by an increase above steady-state levels. This is consistent with the transitory increase in cell-surface density of CD45RA observed at 1-2 days poststimulation. Within 24 h of stimulation the level of high m.w. CD45 mRNA begins to decline permanently, and low m.w. CD45 mRNA is expressed. In mitogen-stimulated cells the $t_{1/2}$ of high and low m.w. CD45 mRNA was estimated at 2.25 h and 3.5 h respectively. The relatively short half-life of high m.w. CD45 mRNA is controlled by a labile degradation pathway that itself may be induced, as suggested by a lag phase prior to the onset of decay of high m.w. CD45 mRNA in stimulated CD45RA$^+$ T cells. Thus, the transition from CD45RA to CD45R0 expression involves a rapid loss of high m.w. CD45 mRNA followed by a more gradual loss of surface CD45RA molecules.

We have proposed that the expression of CD45RA is essential to the survival of thymocytes within the intrathymic generative lineage (10), and shown that progenitor thymocytes express the high m.w. isoform of CD45 (10). We suggest that expression of CD45RA reflects a requirement for certain cellular interactions mediated by CD45RA and its ligand, and/or the nature or strength of signal effected by CD45RA. A switch to CD45R0 expression would then, by default, signal cell death in the thymus. In the periphery, CD45R0$^+$ cells do not die as a result of the switch to low m.w. CD45 expression; rather, they do not require signals provided by CD45RA. A difference in signaling requirements between fully differentiated T cells and thymocytes is not unexpected.

Work designed to test these ideas, and described in this thesis, provides information on the signaling requirements of the most immature cells that can be isolated from human thymus, and demonstrates the ability of CD45RA to transmit activation signals in these cells. The induction of IL-2 and IL-2R gene expression was used as an indicator of functional potential, and correlated with the cell-surface expression of CD45RA. We found that when total thymocytes were enriched for CD45RA$^+$ cells by depletion of CD45R0$^+$ cells, the resulting population had an enhanced ability to express IL-2 and IL-2R mRNA. CD3$^-$4$^-$8$^-$ thymocytes, which are predominantly CD45RA$^+$, expressed IL-2 and IL-2R mRNA in response to PHA and PMA. Since early thymocytes lack the CD3/TCR complex but are responsive to stimulation by PHA, which is thought to act through the TCR, an alternate cell surface molecule must be involved in signal transduction. CD2 and CD45RA are candidates for such structures, since both have been shown to have signaling properties in mature T cells (26,118). Because signaling via CD2 is tightly linked to the CD3 activation pathway in peripheral blood T cells, we speculated that CD2-induced activation might be defective in CD3$^-$ thymocytes and that

CD45RA might be the primary signaling molecule through which PHA mediates cellular activation in the progenitor population. Although we later learned that CD2 does, in fact, transduce activation signals that, in concert with signals mediated through CD28, result in cellular proliferation, these early ideas lead to testing of the ability of CD45RA mAb to augment IL-2 and IL-2R mRNA expression in suboptimally stimulated $CD3^-4^-8^-$ thymocytes. In those experiments we learned that anti-CD45RA mAb, but not an antibody that recognizes a constant CD45 determinant, restored IL-2 and IL-2R gene expression in suboptimally stimulated $CD3^-4^-8^-$ thymocytes. However, CD45RA antibodies were by themselves unable to activate the cells; the additional requirement for suboptimal doses of PHA and PMA suggested that signaling molecules other than CD45RA were also involved in the activation of $CD3^-4^-8^-$ thymocytes.

In order to explore the additional signaling requirements of T cell progenitors, and to determine the influence of CD45RA at later stages in the activation of thymic progenitors, we turned to assays of proliferative responses. We found that, unlike mature peripheral blood T cells which do not respond to CD2-stimulation after CD3 has been modulated from the cell surface, $CD3^-$ progenitor T cells proliferate well to CD2-mediated stimulation in the presence of CD28 mAb. Maximal proliferation of $CD3^-4^-8^-$ thymocytes was obtained using combinations of CD2, CD28 and CD45RA antibodies. Antibodies against common CD45 determinants, however, were strongly inhibitory when covalently linked to anti-CD28, suggesting that the modulatory effects of CD45 molecules may depend on which epitopes are ligated. We suggest that the determining factors in vivo are the natural ligands of CD45.

In vitro activation of early thymocytes through CD2 and CD28 does not lead to the rapid loss of high m.w. CD45 mRNA as it does in mitogen-activated mature peripheral

T cells. This implies prolonged maintenance of CD45RA expression as the progenitors develop. It remains to be determined whether this is an intrinsic property of immature T cells, or a reflection of the method used to stimulate them. However, the point may be irrevelant if in vitro activation via CD2 and CD28 relates to the physiological stimuli in vivo.

If our working hypothesis were correct, $CD3^-4^-8^-$ thymocytes selected for the absence of $CD45R0^+$ cells would be predicted to be the least mature of the progenitor population, since $CD45R0^+$ $CD3^-4^-8^-$ thymocytes would represent those that are committed to cell death. Chapter 5 presents evidence from Northern analyses of TCR-α and -β gene expression that CD45RA is indeed expressed on the least differentiated cells of the $CD3^-4^-8^-$ subset.

All of the data supports our proposal for the role of CD45 in T cell development. But what experiments could be devised to stringently test the hypothesis?
Ultimately it will be necessary to turn to the murine system to do intrathymic transfer experiments using purified populations of $CD45RA^+$ $CD45R0^-$ and $CD45RA^-$ $CD45R0^+$ cells, with the expectation that only $CD45RA^+$ $CD45R0^-$ cells will have reconstitution potential. However, that approach will have to await the development of antibodies against murine CD45RA.

In the human system, strong circumstantial support for our working hypothesis could be gained by separating each of the major thymocyte subsets into $CD45RA^+$ $CD45R0^-$ and $CD45RA^-$ $CD45R0^+$ populations, and testing their functional ability and differentiation potential in vitro. In preliminary experiments, $CD3^-4^-8^-$ thymocytes were cultured without any deliberate stimulus; after 3 days 40% of cells expressed CD3 and/or CD4 and/or CD8. Of the CD3$^-$4$^-$8$^-$ cells remaining, 86% were CD45RA$^+$ CD45R0$^-$, whereas 55% of the CD348$^+$ cells had gained CD45R0. A more detailed analysis of this type will be a valuable approach to further study. In addition, it should be possible to demonstrate that all CD3$^-$4$^-$8$^-$ CD45R0$^+$ cells have rearranged TCR genes. As well, it may be possible in future to test reconstitution potential of human thymocyte subsets in vivo using SCID mice.

In summary, the results show that

- Activated mature T cells initially increase expression of high m.w. CD45 mRNA but the level declines within 24 h. In contrast, in CD3-4-8- thymocytes activated with CD2 and CD28 antibodies, the level of high m.w. CD45 mRNA remains high even at 48 h.

- The $t_{1/2}$ of mRNA encoding high and low m.w. isoforms was estimated at 2.25 h and 3.5 h, respectively. Degradation of high m.w. CD45 mRNA was shown by cycloheximide superinduction to be dependent on a labile factor that may be induced following activation.

- CD45RA transduces signals in CD3$^-$4$^-$8$^-$ thymocytes that result in IL-2 and IL-2R gene expression, and in augmentation of the proliferative response.

- CD3$^-$4$^-$8$^-$ thymocytes that are further depleted of CD45R0$^+$ cells are at an even earlier stage of differentiation than are the CD3$^-$4$^-$8$^-$ thymocytes that express CD45R0.

- Maximal proliferation of CD3$^-$4$^-$8$^-$ thymocytes is achieved by induction through CD2, CD28 and CD45RA.

Together, the data support an essential role for CD45RA in intrathymic development, as postulated (10). Since activated mature T cells almost immediately begin alternate splicing of primary CD45 transcripts that results in a shift to low m.w. CD45 isoform expression, it is necessary to address the question of the mechanism by which activated thymocytes may remain CD45RA$^+$.

We suggest that continuous expression of CD45RA on thymocytes depends on activation via CD28. This proposal is made on the basis of the evidence that mRNA encoding high m.w. CD45 is not lost from CD3$^-$4$^-$8$^-$ thymocytes after activation via CD2 and CD28, and may be subject to some of the same controls as transcripts from other inducible T cell genes. CD28-induced T cell stimulation stabilizes IL-2 and other inducible mRNA's, a phenomenon that may be associated with the presence of AU-rich consensus sequences in the 3' UT region of the mRNA (202). Since CD45 mRNA also contains such sequences, it is theoretically possible that CD28 stabilizes CD45 mRNA as well. This argument may also hold for mature T cells activated through CD28; such a mechanism may provide a means by which antigen-experienced T cells expand their numbers without differentiating into memory cells. Therefore it will be very interesting to examine the CD45 isoform shifts in T cells activated by either CD3 or CD2, in the presence and absence of CD28 stimulation.

The functional role of CD45 will not be fully appreciated until the substrates for the PTPase activity in the cytoplasmic domain, and the ligands for the various isoforms have been identified. CD45 is the most abundant molecule on the T cell surface:

clarification of the interrelationships between CD45 and other cell surface molecules, including CD28, is certain to contribute significantly to our understanding of the mechanisms involved in T cell development.

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